

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
7 September 2001 (07.09.2001)

PCT

(10) International Publication Number
WO 01/64212 A1

(51) International Patent Classification⁷: **A61K 31/23**

(21) International Application Number: **PCT/GB01/00858**

(22) International Filing Date: 28 February 2001 (28.02.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0004895.9 1 March 2000 (01.03.2000) GB
0027989.3 16 November 2000 (16.11.2000) GB

(71) Applicant (for all designated States except US): **UNIVERSITY COLLEGE LONDON [GB/GB]**; Gower Street, London WC1E 6BT (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **BAKER, David [GB/GB]**; Neuroinflammation Group, Institute of Neurology, Department of Neurochemistry, University College London, Wakefield Street, London WC1N 1PJ (GB). **PRYCE, Gareth [GB/GB]**; Neuroinflammation Group, Institute of Neurology, Department of Neurochemistry, University College London, Wakefield Street, London WC1N 1PJ (GB). **DI MARZO, Vincenzo [IT/IT]**; Istituto per la Chimica di Molecole di Interesse Biologico-C.N.R., Via Toiano 6, I-80072 Arco Felice (IT). **BISOGNO, Tiziana [IT/IT]**; Istituto per la Chimica di Molecole di Interesse Biologico-C.N.R., Via Toiano 6, I-80072 Arco

Felice (IT). **DE PETROCELLIS, Luciano [IT/IT]**; Istituto per la Chimica di Molecole di Interesse Biologico-C.N.R., Via Toiano 6, I-80072 Arco Felice (IT).

(74) Agents: **HARDING, Charles, Thomas et al.**; D. Young & Co., 21 New Fetter Lane, London EC4A 1DA (GB).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

36
36
72

WO 01/64212 A1

(54) Title: **MODULATORS OF THE ENDOCANNABINOID UPTAKE AND OF THE VALLINOID RECEPTORS**

(57) Abstract: A method of treatment is described. The method comprises administering to a subject suffering from a muscular disorder a modulator of endocannabinoid and in such an amount to treat said muscular disorder.

MODULATORS OF THE ENDOCANNABINOID UPTAKE AND OF THE VALLINOID RECEPTORS

FIELD OF INVENTION

5

The present invention relates to a modulator.

In particular, the present invention relates to the therapeutic use of a modulator of cannabinoid uptake, in particular endocannabinoid reuptake.

10

More in particular, the present invention relates to the therapeutic use of an inhibitor of endocannabinoid uptake, in particular endocannabinoid reuptake.

15

More in particular, the present invention relates to the therapeutic use of an inhibitor of endocannabinoid reuptake.

BACKGROUND

Chronic relapsing experimental allergic encephalomyelitis (CREAE) is an
20 autoimmune model of multiple sclerosis (MS)¹. Although these diseases are typified by relapsing-remitting paralytic episodes, following CREAE induction by sensitisation to myelin antigens¹, Biozzi ABH mice also developed spasticity and tremor. These symptoms also occur during MS and are difficult to control. This has prompted some patients to self-medicate, and perceive benefit from cannabis use². While these
25 claims have been backed up by small clinical studies, mainly with non-quantifiable outcomes³⁻⁷, the value of cannabis use in MS remains anecdotal.

In addition, while high doses of THC (major psychoactive component of cannabis) can inhibit the development of EAE in rodents^{9,10}, this was attributed to
30 immunosuppression preventing the conditions which lead to the development of paralysis, rather than a direct effect on the paralysis itself^{9,10}. The action of cannabinoids on experimental spasticity and tremor remains uncertain however, because hitherto there have been no behavioural data on effects of cannabinoids in animal models relevant to these symptoms in MS.

35

2

The present invention seeks to provide a means for treating neuromuscular control defects, such as those brought on by neurological disorders – such as MS, Parkinsons disease and Huntingdon's Chorea.

5 SUMMARY ASPECTS OF THE PRESENT INVENTION

We have found that the cannabinoid system can control spasticity and tremors. We have also found that the endogenous system is active and can be manipulated to produce a beneficial therapeutic effect.

10

In accordance with the present invention we have surprisingly found that it is possible to treat (such as cure and/or prevent and/or alleviate) neuromuscular disorders and/or neuromuscular control defects, such as those brought on by neurological disorders – such as MS, Parkinsons disease and Huntingdon's Chorea, Epilepsy, 15 Tourettes' syndrome, bladder spasm – by modulating (in particular inhibiting) part or all of the cannabinoid system, in particular the endocannabinoid system, or one or more components thereof.

20

In one preferred aspect, the inhibition occurs through the action of the agent acting as an inhibitor. In particular, preferably the inhibition may occur through the action of an agent that acts as an endocannabinoid uptake inhibitor.

25

Thus, preferred agents of the present invention may be classified as being cannabinoid inhibitors.

In a preferred aspect, the agents of the present invention are endocannabinoid inhibitors.

30

In a more preferred aspect, the agents of the present invention are endocannabinoid uptake inhibitors.

In a more preferred aspect, the agents of the present invention are endocannabinoid reuptake inhibitors.

35

The present invention relates to methods of treatment using these agents as well as to medicaments comprising same for use in therapy.

In addition, it is believed that the agents of the present invention may also play a role as neuroprotective agents.

5 DETAILED ASPECTS OF THE INVENTION

According to a first aspect of the present invention there is provided the use of a modulator of endocannabinoids in the manufacture of a medicament to treat a muscular disorder.

10

According to a second aspect of the present invention there is provided the use of a modulator of endocannabinoids in the manufacture of a medicament to control spasticity and tremors.

15

According to a third aspect of the present invention there is provided a method of treatment comprising administering to a subject suffering from a muscular disorder a modulator of endocannabinoids and in such an amount to treat said muscular disorder.

20

According to a fourth aspect of the present invention there is provided a method of treatment comprising administering to a subject suffering from a muscular disorder a modulator of endocannabinoids and in such an amount to control spasticity and tremors.

25

According to a fifth aspect of the present invention there is provided a modulator of an endocannabinoid to treat a muscular disorder.

30

According to a sixth aspect of the present invention there is provided a pharmaceutical composition comprising a modulator of an endocannabinoid and optionally a pharmaceutically active carrier, diluent or excipient, said composition for subsequent use to treat a muscular disorder.

35

For ease of reference, these and further aspects of the present invention are now discussed under appropriate section headings. However, the teachings under each section are not necessarily limited to each particular section.

PREFERRED ASPECTS OF THE INVENTION

Preferably, said modulator inhibits the reuptake of an endocannabinoid.

5

Preferably, said modulator does not substantially agonise the CB1 receptor

Preferably, said muscular disorder is a neuromuscular disorder.

10 Preferred agents are presented in the Examples section herein.

ADVANTAGES

15

A key advantage is that the agents of the present invention can be used to treat neuromuscular disorders and/or act as neuroprotectants.

In addition, it is believed that the agents of the present invention may not have psychoactive effects. This has tremendous clinical implications.

20

MUSCULAR DISORDER

25

The term "muscular disorder" is used in a broad sense to cover any muscular disorder or disease, in particular a neurological disorder or disease, more in particular a neurodegenerative disease or an adverse condition involving neuromuscular control. Thus, in addition to each of the afore-mentioned disorders (e.g. CREA, MS, spasticity, Parkinson's disease, Huntington's Chorea, Spinal cord injury Epilepsy, Tourettes' syndrome, bladder and spasm) the present invention also has applications in other fields where tremor or muscle spasm is present or is manifested – such as incontinence, asthma, bronchial spasms, hic-coughs etc.

30

SPASTICITY

35

Spasticity is a complicating sign in multiple sclerosis and also develops in a model of chronic relapsing experimental autoimmune encephalomyelitis (CREAE) in mice. In areas associated with nerve damage, increased levels of the endocannabinoids, anandamide (arachidonylethanolamide, AEA) and 2-arachidonoyl glycerol (2-AG),

5
and of the AEA congener, palmitoylethanolamide (PEA), were detected, whereas comparable levels of these compounds were found in normal and non-spastic CREAE mice. Whilst exogenously administered endocannabinoids and PEA ameliorate spasticity, selective inhibitors of endocannabinoid re-uptake and
5 hydrolysis, probably through the enhancement of endogenous levels of AEA, and, possibly, 2-arachidonoyl glycerol, significantly ameliorated spasticity, to an extent comparable to that observed previously with potent cannabinoid receptor agonists. The studies presented herein provide definitive evidence for the tonic control of spasticity by the endocannabinoid system and open new horizons to therapy of
10 multiple sclerosis, and other neuromuscular diseases, based on agents modulating endocannabinoid levels and action, which exhibit little psychotropic activity. These studies also demonstrate that the endocannabinoid system exhibits tonic control of spasticity in an MS-like condition.

15 CANNABINOID

A cannabinoid is an entity that is capable of binding to a cannabinoid receptor, in particular CB1 and/or CB2. Typical cannabinoids include the 30 or so derivatives of 2-(2-isopropyl-5-methylphenyl)-5-pentylresorcinol that are found in the Indian hemp,
20 *Cannabis sativa*, among which are those responsible for the narcotic actions of the plant and its extracts. Examples of cannabinoids are cannabidiol, cannabinol, *trans*- Δ^9 -tetrahydrocannabinol, *trans*- Δ^8 -tetrahydrocannabinol, and Δ^9 -tetrahydrocannabinolic acid. Other examples of cannabinoids include anandamide, methanandamide and R(+)-WIN55,212

25

ENDOCANNABINOID

This term means a cannabinoid that exists naturally in the body – as opposed to an exogeneously supplied cannabinoid. Endocannabinoids are discussed by Di Marzo
30 (1998) *Biochimica et Biophysica Acta* vol 1392 pages 153-175 (the contents of which are incorporated herein by reference).

An example of an endocannabinoid is anandamide. Teachings on this entity and anandamide amidase may be found in US-A-5874459. This document teaches the
35 use of anandamide amidase inhibitors as analgesic agents.

MODULATION OF REUPTAKE OF AN ENDOCANNABINOID

In one aspect of the present invention the agent of the present invention acts as a
5 modulator of the reuptake system/mechanism of an endocannabinoid.

It is known some cannabinoids can be taken up by intact cells, when they may be
hydrolysed. By way of example it has been reported that the termination of activity of
arachidonoyl ethanolamide (AEA) – which is better known as anandamide and which is
10 known to act as an endogenous CB1 receptor agonist (Devane *et al* 1992 Science
vol 258 pages 1946-1949) - by cells is as a result of a two-step process including: (i)
re-uptake by intact cells via the 'anandamide membrane transporter' (AMT) and (ii)
hydrolysis of the amide bond, catalysed by the enzyme 'fatty acid amide hydrolase'
(FAAH) (see Deutsch *et al* 1993 Biochem, Pharmacol vol 46 791-796; Di Marzo *et al*
15 1994 Nature vol 372 pages 686-691; Hillard *et al* 1997 J Neurochem vol 69 pages
631-638; Bisogno *et al* 1997 J Biol Chem vol 272 pages 3315-3323; Di Marzo *et al*
(1999) Current Medicinal Chemistry vol 6 pages 721-744 - the contents of each of
which are incorporated herein by reference).

20 The present invention is concerned with modulation, in particular inhibition, of the re-
uptake step of certain cannabinoids, in particular endocannabinoids. This represents
a significant departure from hitherto approaches to treating muscle disorders.

INHIBITOR

25

In a preferred aspect, the agent of the present invention acts as an inhibitor of the re-
uptake of an endocannabinoid.

30 The term "inhibitor" as used herein with respect to the agent of the present invention
means an agent that can reduce and/or eliminate and/or prevent the re-uptake of an
endocannabinoid by an intact cell. This inhibitory action may be direct or indirect –
providing the end result is an inhibition of endocannabinoid re-uptake. Here, the
inhibitor may act as an agonist or as an antagonist.

PSYCHOACTIVE EFFECTS

In order to reduce any psychoactive effects, we believe that the agents of the present invention should not substantially agonise the cannabinoid receptor CB1.

CANNABINOID RECEPTOR

A cannabinoid receptor is any one or more of several membrane proteins that bind cannabinol and structurally similar compounds and mediate their intracellular action.

Two receptors for the psychoactive ingredient of marijuana Δ^9 -tetrahydrocannabinol (THC), the CB1 and CB2 cannabinoid receptors, have been found (Pertwee 1997 Pharmacol Ther vol 74 129-180).

Both of these receptors are seven-transmembrane-domain G-protein-coupled receptors. CB1 receptors are found in brain and testis. CB2 receptors are found in spleen and not in brain.

For both types of receptor arachidonylethanolamide (anandamide) is a putative endogenous ligand and both types are negatively coupled to adenylate cyclase decreasing intracellular cyclic AMP levels. Examples of sequences for such receptors are from *Mus musculus* – and which include: CB1, database code CB1R_MOUSE, 473 amino acids (52.94 kDa); CB2, database code CB2R_MOUSE, 347 amino acids (38.21 kDa). More details on CB1 and CB2 now follow.

CANNABINOID RECEPTOR 1 (CB1 or CNR1)

Background teachings on CB1 have been presented by Victor A. McKusick *et al* on <http://www.ncbi.nlm.nih.gov/Omim>. The following information concerning CB1 has been extracted from that source.

The cannabinoids are psychoactive ingredients of marijuana, principally delta-9-tetrahydrocannabinol, as well as the synthetic analogs. Matsuda *et al.* (1990) cloned a cannabinoid receptor from a rat brain. Using a cosmid clone of the entire coding sequence of the human gene, Modi and Bonner (1991) mapped the human CNR locus to

8

6q14-q15 by in situ hybridization. Gerard et al. (1991) isolated a cDNA encoding a cannabinoid receptor from a human brain stem cDNA library. The deduced amino acid sequence encoded a protein of 472 residues which shared 97.3% identity with the rat cannabinoid receptor cloned by Matsuda et al. (1990). They provided evidence for the existence of an identical cannabinoid receptor expressed in human testis. Hoehe et al. (1991) determined the genomic localization of the CNR gene by combination of genetic linkage mapping and chromosomal in situ hybridization. Close linkage was suggested with CGA which is located at 6q21.1-q23; maximum lod = 2.71 at theta = 0.0. Moreover, CNR was linked to markers that define locus D6Z1, a sequence localized exclusively to centromeres of all chromosomes and enriched on chromosome 6. Ledent et al. (1999) investigated the function of the central cannabinoid receptor (CB1) by disrupting the gene in mice. Mutant mice did not respond to cannabinoid drugs, demonstrating the exclusive role of CB1 in mediating analgesia, reinforcement, hypothermia, hypolocomotion, and hypotension.

CANNABINOID RECEPTOR 2 (CB2 or CNR2)

Background teachings on CB1 have been presented by Victor A. McKusick *et al* on <http://www.ncbi.nlm.nih.gov/Omim>. The following information concerning CB2 has been extracted from that source.

In addition to its renowned psychoactive properties, marijuana, or its major active cannabinoid ingredient, delta-9-tetrahydrocannabinol, exerts analgesic, antiinflammatory, immunosuppressive, anticonvulsive, and antiemetic effects as well as the alleviation of intraocular pressure in glaucoma. The G protein-coupled cannabinoid receptor-1 (CNR1; 114610), which is expressed in brain but not in the periphery, apart from low levels in testis, does not readily account for the nonpsychoactive effects of cannabinoids.

Using PCR with degenerate primers to screen a promyelocytic leukemia cell cDNA library, Munro et al. (1993) obtained a cDNA encoding CNR2, which the authors called CX5. Sequence analysis predicted that the deduced 360-amino acid 7-transmembrane-spanning protein has 44% amino acid identity with CNR1 overall and 68% identity with the transmembrane residues proposed to confer ligand specificity. Binding analysis determined that CNR2 encodes a high-affinity receptor for cannabinoids, with higher affinity than CNR1 for cannabinol. Northern blot analysis revealed that the expression of 2.5- and 5.0-kb transcripts in the HL60 myeloid cell line increases on myeloid, or granulocyte, differentiation. Using the rat CX5 homolog, Munro et al. (1993) found that the 2.5-kb transcript is expressed in spleen but not in brain, kidney, lung, thymus, liver, or nasal epithelium. In situ hybridization analysis demonstrated expression in splenic marginal zones. PCR analysis detected CNR2 expression in purified splenic macrophages but not in CD5+ T cells. Munro et al. (1993) speculated that the location of CNR2 suggests that its endogenous ligand should have an immunomodulatory role.

The International Radiation Hybrid Mapping Consortium mapped the CNR2 gene to chromosome 1 (stSG90).

5 ASSAY

The present invention uses – and also encompasses - an assay, wherein said assay is used to screen for agents that can modulate endocannabinoid reuptake, in particular inhibit endocannabinoid reuptake and/or modulate a vallinoid receptor, in
10 particular agonise VR1. Details on such assays are presented later.

In addition, the present invention also encompasses an assay, wherein said assay is used to screen for agents that do not substantially agonise the cannabinoid receptor CB1.

15

In each of these assays, one or more of appropriate targets - such as an amino acid sequence and/or nucleotide sequence - may be used for identifying an agent capable of modulating endocannabinoid reuptake and/or not agonising the cannabinoid receptor CB1. The target employed in such a test may be free in solution, affixed to
20 a solid support, borne on a cell surface, or located intracellularly. The abolition of target activity or the formation of binding complexes between the target and the agent being tested may be measured.

The assay of the present invention may be a screen, whereby a number of agents
25 are tested. In one aspect, the assay method of the present invention is a high through put screen.

Techniques for drug screening may be based on the method described in Geysen, European Patent Application 84/03564, published on September 13, 1984. In
30 summary, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with a suitable target or fragment thereof and washed. Bound entities are then detected - such as by appropriately adapting methods well known in the art. A purified target can also be coated directly onto plates for use in a
35 drug screening techniques. Alternatively, non-neutralising antibodies can be used to capture the peptide and immobilise it on a solid support.

10

This invention also contemplates the use of competitive drug screening assays in which neutralising antibodies capable of binding a target specifically compete with a test compound for binding to a target.

- 5 Another technique for screening provides for high throughput screening (HTS) of agents having suitable binding affinity to the substances and is based upon the method described in detail in WO-A-84/03564.

10 It is expected that the assay methods of the present invention will be suitable for both small and large-scale screening of test compounds as well as in quantitative assays.

In one preferred aspect, the present invention relates to a method of identifying agents that selectively modulate endocannabinoid reuptake and which do not substantially agonise the cannabinoid receptor CB1.

15

In a preferred aspect, the assay of the present invention utilises cells that display CB1 on their surface. These cells may be isolated from a subject possessing such cells. However, preferably, the cells are prepared by transfecting cells so that upon transfect those cells display on their surface CB1.

20

REPORTERS

A wide variety of reporters may be used in the assay methods (as well as screens) of the present invention with preferred reporters providing conveniently detectable
25 signals (eg. by spectroscopy). By way of example, a reporter gene may encode an enzyme which catalyses a reaction which alters light absorption properties.

Other protocols include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). A two-site,
30 monoclonal-based immunoassay utilising monoclonal antibodies reactive to two non-interfering epitopes may even be used. These and other assays are described, among other places, in Hampton R et al (1990, Serological Methods, A Laboratory Manual, APS Press, St Paul MN) and Maddox DE et al (1983, J Exp Med 15 8:121 1).

35

Examples of reporter molecules include but are not limited to (galactosidase,

11

invertase, green fluorescent protein, luciferase, chloramphenicol, acetyltransferase, (glucuronidase, exo-glucanase and glucoamylase. Alternatively, radiolabelled or fluorescent tag-labelled nucleotides can be incorporated into nascent transcripts which are then identified when bound to oligonucleotide probes.

5

By way of further examples, a number of companies such as Pharmacia Biotech (Piscataway, NJ), Promega (Madison, WI), and US Biochemical Corp (Cleveland, OH) supply commercial kits and protocols for assay procedures. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US-A-3817837; US-A-3850752; US-A-3939350; US-A-3996345; US-A-4277437; US-A-4275149 and US-A-4366241.

15 TRANSPORTER ASSAY

Details on an assay that may be used to determine if a compound could be used as a beneficial modulator of endocannabinoid uptake may be found in Petrocellis *et al* (2000) FEBS Letter 483 52-56. The relevant Information about that assay from that reference now follows. It is to be noted that other suitable assay(s) may be used.

AEA transporter assays

The effect of the compound on the uptake of AEA by RBL-2H3 cells was described by a modification of the method described previously (6,12,13) and analogous to the protocol described in (5,16) except for the use of higher concentration (4 μ M) of [¹⁴C]AEA. Cells were incubated with [¹⁴C]AEA for 5 min at 37°C, in the presence or absence of varying concentrations of the inhibitors. Residual [¹⁴C]AEA in the incubation media after extraction with CHCl₃/ CH₃OH 2:1 (by vol) determined by scintillation counting was used as a measure of AEA that was taken up by cells. We applied the same protocol also to C6 rat glioma cells, which also contain membrane transporter for AEA [3]. Data are expressed as concentration exerting 50% inhibition of AEA uptake (IC₅₀) calculated with GraphPad. [The relevant references may be obtained from the paper itself.]

CB1 RECEPTOR AND CB2 RECEPTOR BINDING ASSAY

Details on a CB1 receptor binding assay and a CB2 receptor binding assay may be found in Petrocellis *et al* (2000) FEBS Letter 483 52-56. The relevant Information about those assays from that reference now follows now follow. Other assays may

be used.

CB₁ and CB₂ receptor binding assays

5 Displacement assays for CB₁ receptors were carried out by using ³H]SR141716A (0.4nM, 55 Ci/mmol, Amersham) as the high affinity ligand, and the filtration technique previously described [12-14], on membrane preparations (0.4 mg/tube) from frozen male CD rat brains (Charles River Italia) and in the presence of 100 µM PMSF. Specific binding was calculated with 1 µM SR 14176A (a gift from Sanofi Recherche, France) and was 84.0%.
10 The spleen from CD rats were used to prepare membranes (0.4 mg/tube) to carry out CB₂ binding assays by using [³H]WIN55,212-2 (0.8nM, 50.8 Ci/mmol, NEN-Dupont) as described previously [14], and again in the presence of 100 µM PMSF. Specific binding was calculated with 1 µM HU-348 (a gift from Prof. R. Mechoulam and Pharmos) and was 75.0%. In all cases, K_i values were calculated by applying the Cheng-prusoff equation to the IC₅₀ values (obtained by GraphPad) for the displacement of the bound radioligand by increasing concentrations of the test compounds. [Details on the specific references may be found in the document itself.]
20

HOST CELLS

Polynucleotides for use in the present invention – such as for use as targets or for
25 expressing targets - may be introduced into host cells.

The term "host cell" - in relation to the present invention includes any cell that could comprise the target for the agent of the present invention.

30 Here, polynucleotides may be introduced into prokaryotic cells or eukaryotic cells, for example yeast, insect or mammalian cells.

Polynucleotides of the invention may introduced into suitable host cells using a variety of techniques known in the art, such as transfection, transformation and
35 electroporation. For example, it is possible to cause transformation with recombinant viral vectors such as retroviruses, herpes simplex viruses and adenoviruses, direct injection of nucleic acids and biolistic transformation.

Thus, a further embodiment of the present invention provides host cells transformed
40 or transfected with a polynucleotide that is or expresses the target of the present invention. Preferably said polynucleotide is carried in a vector for the replication and expression of polynucleotides that are to be the target or are to express the target.

The cells will be chosen to be compatible with the said vector and may for example be prokaryotic (for example bacterial), fungal, yeast or plant cells.

5 The gram negative bacterium *E. coli* is widely used as a host for heterologous gene expression. However, large amounts of heterologous protein tend to accumulate inside the cell. Subsequent purification of the desired protein from the bulk of *E. coli* intracellular proteins can sometimes be difficult.

10 In contrast to *E. coli*, bacteria from the genus *Bacillus* are very suitable as heterologous hosts because of their capability to secrete proteins into the culture medium. Other bacteria suitable as hosts are those from the genera *Streptomyces* and *Pseudomonas*.

15 Depending on the nature of the polynucleotide encoding the polypeptide of the present invention, and/or the desirability for further processing of the expressed protein, eukaryotic hosts such as yeasts or other fungi may be preferred. In general, yeast cells are preferred over fungal cells because they are easier to manipulate. However, some proteins are either poorly secreted from the yeast cell, or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these
20 instances, a different fungal host organism should be selected.

Examples of suitable expression hosts within the scope of the present invention are fungi such as *Aspergillus* species (such as those described in EP-A-0184438 and EP-A-0284603) and *Trichoderma* species; bacteria such as *Bacillus* species (such as
25 those described in EP-A-0134048 and EP-A-0253455), *Streptomyces* species and *Pseudomonas* species; and yeasts such as *Kluyveromyces* species (such as those described in EP-A-0096430 and EP-A-0301670) and *Saccharomyces* species. By way of example, typical expression hosts may be selected from *Aspergillus niger*, *Aspergillus niger* var. *tubigenis*, *Aspergillus niger* var. *awamori*, *Aspergillus aculeatis*,
30 *Aspergillus nidulans*, *Aspergillus oryzae*, *Trichoderma reesei*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Kluyveromyces lactis* and *Saccharomyces cerevisiae*.

35 Polypeptides that are extensively modified may require correct processing to complete their function. In those instances, mammalian cell expression systems (such as HEK-293, CHO, HeLA) are required, and the polypeptides are expressed

14

either intracellularly, on the cell membranes, or secreted in the culture media if preceded by an appropriate leader sequence.

The use of suitable host cells - such as yeast, fungal, plant and mammalian host cells - may provide for post-translational modifications (e.g. myristoylation, glycosylation, truncation, lipidation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the present invention.

10 ORGANISM

The term "organism" in relation to the present invention includes any organism that could comprise the target according to the present invention and/or products obtained therefrom. Examples of organisms may include a fungus, yeast or a plant.

15

The term "transgenic organism" in relation to the present invention includes any organism that comprises the target according to the present invention and/or products obtained.

20 TRANSFORMATION OF HOST CELLS/HOST ORGANISMS

As indicated earlier, the host organism can be a prokaryotic or a eukaryotic organism. Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*.

25 Teachings on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook *et al* (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press) and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc.

30 If a prokaryotic host is used then the nucleotide sequence may need to be suitably modified before transformation - such as by removal of introns.

In another embodiment the transgenic organism can be a yeast. In this regard, yeast have also been widely used as a vehicle for heterologous gene expression. The species *Saccharomyces cerevisiae* has a long history of industrial use, including its use for heterologous gene expression. Expression of heterologous genes in *Saccharomyces cerevisiae* has been reviewed by Goodey *et al* (1987, Yeast Biotechnology, D R Berry *et al*, eds, pp 401-429, Allen and Unwin, London) and by

35

King et al (1989, Molecular and Cell Biology of Yeasts, E F Walton and G T Yarronton, eds, pp 107-133, Blackie, Glasgow).

For several reasons *Saccharomyces cerevisiae* is well suited for heterologous gene expression. First, it is non-pathogenic to humans and it is incapable of producing certain endotoxins. Second, it has a long history of safe use following centuries of commercial exploitation for various purposes. This has led to wide public acceptability. Third, the extensive commercial use and research devoted to the organism has resulted in a wealth of knowledge about the genetics and physiology as well as large-scale fermentation characteristics of *Saccharomyces cerevisiae*.

A review of the principles of heterologous gene expression in *Saccharomyces cerevisiae* and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a vehicle for the expression of heterologous genes", Yeasts, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

Several types of yeast vectors are available, including integrative vectors, which require recombination with the host genome for their maintenance, and autonomously replicating plasmid vectors.

In order to prepare the transgenic *Saccharomyces*, expression constructs are prepared by inserting the nucleotide sequence of the present invention into a construct designed for expression in yeast. Several types of constructs used for heterologous expression have been developed. The constructs contain a promoter active in yeast fused to the nucleotide sequence of the present invention, usually a promoter of yeast origin, such as the GAL1 promoter, is used. Usually a signal sequence of yeast origin, such as the sequence encoding the SUC2 signal peptide, is used. A terminator active in yeast ends the expression system.

For the transformation of yeast several transformation protocols have been developed. For example, a transgenic *Saccharomyces* according to the present invention can be prepared by following the teachings of Hinnen et al (1978, Proceedings of the National Academy of Sciences of the USA 75, 1929); Beggs, J D (1978, Nature, London, 275, 104); and Ito, H et al (1983, J Bacteriology 153, 163-168).

16

The transformed yeast cells are selected using various selective markers. Among the markers used for transformation are a number of auxotrophic markers such as LEU2, HIS4 and TRP1, and dominant antibiotic resistance markers such as aminoglycoside antibiotic markers, eg G418.

5

Another host organism is a plant. The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material. Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). Further teachings on plant transformation may be found in EP-A-0449375.

15

Further hosts suitable for the nucleotide sequence of the present invention include higher eukaryotic cells, such as insect cells or vertebrate cells, particularly mammalian cells, including human cells, or nucleated cells from other multicellular organisms. In recent years propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are epithelial or fibroblastic cell lines such as Chinese hamster ovary (CHO) cells, NIH 3T3 cells, HeLa cells or 293T cells.

20

The nucleotide sequence of the present invention may be stably incorporated into host cells or may be transiently expressed using methods known in the art. By way of example, stably transfected mammalian cells may be prepared by transfecting cells with an expression vector having a selectable marker gene, and growing the transfected cells under conditions selective for cells expressing the marker gene. To prepare transient transfectants, mammalian cells are transfected with a reporter gene to monitor transfection efficiency.

30

To produce such stably or transiently transfected cells, the cells should be transfected with a sufficient amount of the nucleotide sequence of the present invention. The precise amounts of the nucleotide sequence of the present invention may be empirically determined and optimised for a particular cell and assay.

35

Thus, the present invention also provides a method of transforming a host cell with a nucleotide sequence that is to be the target or is to express the target. Host cells transformed with the nucleotide sequence may be cultured under conditions suitable for the expression of the encoded protein. The protein produced by a recombinant cell may be displayed on the surface of the cell. If desired, and as will be understood by those of skill in the art, expression vectors containing coding sequences can be designed with signal sequences which direct secretion of the coding sequences through a particular prokaryotic or eukaryotic cell membrane. Other recombinant constructions may join the coding sequence to nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins (Kroll DJ et al (1993) DNA Cell Biol 12:441-53).

AGENT

The agent of the present invention may be an organic compound or other chemical. The agent can be an amino acid sequence or a chemical derivative thereof, or a combination thereof. The agent may even be a nucleotide sequence - which may be a sense sequence or an anti-sense sequence. The agent may even be an antibody.

Typically, the agents will be organic compounds.

Examples of suitable agents are presented in the Examples Section herein. Further examples may be selected from appropriate compounds mentioned in Petrocellis et al (2000) FEBS Letter 483 52-56 (especially Table 1 thereof).

DUAL EFFECT

In some cases the agent of the present invention may behave as both an inhibitor of endocannabinoid uptake and endocannabinoid hydrolysis and/or a vanilloid receptor agonist.

THERAPY

The agents of the present invention, even those identified by the assay method of the present invention, may be used as therapeutic agents - i.e. in therapy applications. In particular, the therapy relates to treating muscular disorders and/or neuroprotection.

The term "therapy" includes curative effects, alleviation effects, and prophylactic effects.

- 5 The therapy may be on humans or animals.

PHARMACEUTICAL COMPOSITIONS

10 The present invention also provides a pharmaceutical composition comprising administering a therapeutically effective amount of the agent of the present invention and a pharmaceutically acceptable carrier, diluent or excipients (including combinations thereof).

15 The pharmaceutical compositions may be for human or animal usage in human and veterinary medicine and will typically comprise any one or more of a pharmaceutically acceptable diluent, carrier, or excipient. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985). The choice of pharmaceutical carrier, excipient or diluent can
20 be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s).

25 Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. Examples of preservatives include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. Antioxidants and suspending agents may be also used.

30 There may be different composition/formulation requirements dependent on the different delivery systems. By way of example, the pharmaceutical composition of the present invention may be formulated to be delivered using a mini-pump or by a mucosal route, for example, as a nasal spray or aerosol for inhalation or ingestible solution, or parenterally in which the composition is formulated by an injectable form,
35 for delivery, by, for example, an intravenous, intramuscular or subcutaneous route. Alternatively, the formulation may be designed to be delivered by both routes.

Where the agent is to be delivered mucosally through the gastrointestinal mucosa, it should be able to remain stable during transit through the gastrointestinal tract; for example, it should be resistant to proteolytic degradation, stable at acid pH and
5 resistant to the detergent effects of bile.

Where appropriate, the pharmaceutical compositions can be administered by inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form
10 of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intravenously, intramuscularly or subcutaneously. For
parenteral administration, the compositions may be best used in the form of a sterile
15 aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

20 PHARMACEUTICAL COMBINATIONS

The agent of the present invention may be administered with one or more other pharmaceutically active substances.

25 In the combinations of the present invention, the agent of the present invention and other therapeutic active agents may be administered in terms of dosage forms either separately or in conjunction with each other, and in terms of their time of administration, either serially or simultaneously. Thus, the administration of one component agent may be prior to, concurrent with, or subsequent to the
30 administration of the other component agent(s).

By way of example, the other component agent may be a compound already known to have a beneficial effect vis-à-vis muscular disorder. By way of further example, the other component agent may be a compound may be a modulator of a vanilloid
35 receptor, such as vanilloid receptor VR1. In particular, the other component agent may be a compound may be an agonist of a vanilloid receptor, such as vanilloid

receptor VR1.

ADDITIONAL PHARMACEUTICAL ACTIVITY

5 The agent of the present invention may have at least one additional beneficial therapeutic effect. By way of example, the compound itself may also be capable of acting as modulator of a vanilloid receptor, such as vanilloid receptor VR1. In particular, the compound may be capable of acting as an agonist of a vanilloid receptor, such as vanilloid receptor VR1. Results for such compounds are presented
10 in the Experimental Section herein.

MODULATION OF VANILLOID RECEPTOR

As indicated above, the modulator of the present invention may be capable of acting
15 as and/or be used with a modulator of a vanilloid receptor – in particular an agonist of the vanilloid receptor VR1.

Details on the vanilloid receptor VR1 and modulators thereof (especially agonists thereof) may be found in are presented in the following documents: Szallasi and
20 Blumberg (1999) Pharmacological Reviews vol 51 No. 2 pages 159-211; Petrocellis *et al* (2000) FEBS Letter 483 52-56; Di Marzo *et al* (2000) European Journal of Pharmacology vol 406 pages 363-374; Zygmunt *et al* (1999) Nature vol 400 pages 452-456; WO-A-00/16756; Melck *et al* (1999) Biochemical and Biophysical Res. Comm vol 262 pages 275-284; and Di Marzo *et al* (1998) FEBS Letters vol 436
25 pages 449-454 (the contents of each of which are incorporated herein by reference).

More details on the vanilloid receptor VR1 have been presented by Victor A. McKusick *et al* on <http://www.ncbi.nlm.nih.gov/Omim>. The following information concerning VR1 has been extracted from that source.

30

35

Capsaicin, the main pungent ingredient in 'hot' chili peppers, elicits a sensation of burning pain by selectively activating sensory neurons that convey information about noxious stimuli to the central nervous system. Caterina et al. (1997) used an expression cloning strategy based on calcium influx to isolate a functional cDNA encoding a capsaicin receptor from rat sensory neurons. The rat cDNA encodes an 838-amino acid polypeptide with a predicted relative mass of 95,000 and a hydrophobicity profile suggestive of a 6-transmembrane domain-containing receptor. They showed that this receptor is a nonselective cation channel that is structurally related to members of the *Drosophila* TRP family of ion channels. The cloned

capsaicin receptor is also activated by increases in temperature in the noxious range, suggesting that it functions as a transducer of painful thermal stimuli in vivo. Because a vanilloid moiety constitutes an essential chemical component of capsaicin and resiniferatoxin structures, the proposed site of action of these compounds is generally referred to as the vanilloid receptor. Accordingly, Caterina et al. (1997) named their cloned cDNA VR1, for 'vanilloid receptor subtype 1.' An expressed sequence tag (EST) database search revealed several human clones with a high degree of similarity to VR1 at both the DNA and predicted amino acid sequence levels.

Capsaicin, the compound responsible for the oral burn of chili pepper, is more intensively 'hot' in PTC/PROP tasters (171200) than in nontasters (Bartoshuk et al., 1994). Repeated exposure to capsaicin (over the course of days) decreases the overall burn intensity (Stevenson and Prescott, 1994). This may explain why frequent consumers of chili are less sensitive to its perceived burn (Stevenson and Yeomans, 1993). Other pungent spices that are structurally related to capsaicin include piperine (from black pepper) and zingerone (from ginger). Prescott and Stevenson (1996) observed that the frequent use of chili decreased the psychophysical response to zingerone, suggesting that the 2 compounds act through a common mechanism. Experiments in rodents showed that capsaicin, zingerone, and piperine bind to different subtypes of a common receptor (Liu and Simon, 1996).

Caterina et al. (2000) generated mice deficient in VR1 by targeted disruption. VR1 $-/-$ mice were viable, fertile, and largely indistinguishable from wildtype littermates. Caterina et al. (2000) demonstrated that sensory neurons from mice lacking VR1 are severely deficient in their responses to vanilloid compounds, protons, or heat greater than 43 degrees C. VR1 $-/-$ mice showed normal responses to noxious mechanical stimuli but exhibited no vanilloid-evoked pain behavior, were impaired in the detection of painful heat, and showed little thermal hypersensitivity in the setting of inflammation. Thus, Caterina et al. (2000) concluded that VR1 is essential for selective modalities of pain sensation and for tissue injury-induced thermal hyperalgesia.

Hence, further aspects the present invention relate to:

Use of a modulator of an endocannabinoid according to the present invention in the manufacture of a medicament to treat a muscular disorder; wherein said modulator is also a modulator of a vanilloid receptor and/or wherein said modulator is used in conjunction with a modulator of a vanilloid receptor.

A method of treatment comprising administering to a subject suffering from a muscular disorder a modulator of an endocannabinoid according to the present invention and in such an amount to treat said muscular disorder; wherein said modulator is also a modulator of a vanilloid receptor and/or wherein said modulator is used in conjunction with a modulator of a vanilloid receptor.

22

A method of treatment comprising administering to a subject suffering from a muscular disorder a modulator of endocannabinoid according to the present invention and in such an amount to control spasticity and tremors wherein said modulator is also a modulator of a vanilloid receptor and/or wherein said modulator is used in conjunction with a modulator of a vanilloid receptor.

A modulator of an endocannabinoid according to the present invention to treat a muscular disorder wherein said modulator is also a modulator of a vanilloid receptor and/or wherein said modulator is used in conjunction with a modulator of a vanilloid receptor.

A pharmaceutical composition comprising a modulator of an endocannabinoid according to the present invention and optionally a pharmaceutically active carrier, diluent or excipient, said composition for subsequent use to treat a muscular disorder wherein said modulator is also a modulator of a vanilloid receptor and/or wherein said modulator is used in conjunction with a modulator of a vanilloid receptor.

For some embodiments, a preferred agent for use in the present invention may be a hybrid of entity that possesses an inhibitory action against endocannabinoid re-uptake and an entity that possesses an agonistic effect on VR1. Examples of such agents are mentioned in Petrocellis *et al* (2000) FEBS Letter 483 52-56; Di Marzo *et al* (2000) European Journal of Pharmacology vol 406 pages 363-374 (the contents of which are incorporated herein by reference).

An example of a preferred agent that can act as a modulator according to the present invention is Arvanil (see Petrocellis *et al* (2000) FEBS Letter 483 52-56; Di Marzo *et al* (2000) European Journal of Pharmacology vol 406 pages 363-374.

Highly surprisingly we have found that it is possible to treat muscle disorder using a modulator of a vanilloid receptor, in particular using an agonist of VR1.

Use of a modulator of a vanilloid receptor in the manufacture of a medicament to treat a muscular disorder, preferably wherein said muscular disorder is a neuromuscular disorder.

23

Use of a modulator of a vanilloid receptor in the manufacture of a medicament to control spasticity and tremors.

5 A method of treatment comprising administering to a subject suffering from a muscular disorder a modulator of a vanilloid receptor and in such an amount to treat said muscular disorder, preferably a neuromuscular disorder.

10 A method of treatment comprising administering to a subject suffering from a muscular disorder a modulator of a vanilloid receptor and in such an amount to control spasticity and tremors.

A modulator of a vanilloid receptor to treat a muscular disorder.

15 A pharmaceutical composition comprising a modulator of a vanilloid receptor and optionally a pharmaceutically active carrier, diluent or excipient, said composition for subsequent use to treat a muscular disorder.

Results for such compounds are shown in Figure 11 and Figure 12.

20 Preferably said modulator of vanilloid receptor inhibits the reuptake of an endocannabinoid.

VANILLOID RECEPTOR BINDING ASSAY

25 Details on a vanilloid receptor binding assay may be found in V Di Marzo *et al* (2000) European Journal of Pharmacology 406 363-374. Information about that assay from that document is now presented below.

Vanilloid Receptor VR1 Binding Assays

30

35

Chinese hamster ovary (CHO/rVR, cells were generated as described previously (Szallasi *et al.*, 1999). A 2.7-KB DNA with verified sequence identical to rVR₁ (Caterina *et al.*, 1997) was subcloned into pUHG2102-3 (Clontech, Palo Alto, CA) for recombinant expression in CHO cells containing pTet Off regulator plasmid (Clontech). Ninety percent confluent cells were washed phosphate-buffered saline, harvested in phosphate-buffered saline containing 5mM EDTA, and pelleted by gentle centrifugation to be stored at -80°C until assayed. Binding studies with [³H]resiniferatoxin (37 Ci/mmol Chemical Synthesis and Analysis Laboratory, NCI-FCRDC, Frederick, MD)

were carried out according to a published protocol (Szallasi et al., 1999). Binding assay were set up on ice and contained approximately 100,00 CHO/VR₁ cells, 0.25 mg/ml bovine serum albumin (Cohn fraction V, Sigma), and [³H]resiniferatoxin. The final volume was adjusted to 1000 µl with a buffer containing (in mM) KC15, NaCl 5.8, CaCl₂ 0.75, MgCl₂ 2, and HEPES 10; pH 7.4. Non-specific binding was determined in the presence of 100 nM non-radioactive resiniferatoxin. The binding reaction was terminated after a 30-min incubation at 37°C by cooling the tubes on ice. Bovine α 1-acid glycoprotein (100 µg/tube) was added to reduce non-specific binding (Szallasi et al., 1992). Membrane-bound resiniferatoxin was separated from the free as well as α 1-acid glycoprotein-bound resiniferatoxin by rapid centrifugation at 4°C, and the radioactivity was determined by scintillation counting. Competition experiments were out with various concentrations of each analogue in the presence of 100 pM [³H]resiniferatoxin (the approximated K_d value). IC₅₀ values were calculated by the curvilinear regression program LIGAND (Biosoft, Ferguson, MO). K_i values were obtained by the corresponding IC₅₀ by using the Cheng-Prussoff equation and a K_d value of 100 pM.

ADMINISTRATION

Typically, a physician will determine the actual dosage which will be most suitable for an individual subject and it will vary with the age, weight and response of the particular patient. The dosages below are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited.

The compositions of the present invention may be administered by direct injection.

The composition may be formulated for parenteral, mucosal, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration. Depending upon the need, the agent may be administered at a dose of from 0.01 to 30 mg/kg body weight, such as from 0.1 to 10 mg/kg, more preferably from 0.1 to 1 mg/kg body weight.

By way of further example, the agents of the present invention may be administered in accordance with a regimen of 1 to 4 (or more) times per day, preferably once or twice per day. The specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors including the activity of the specific compound employed, the metabolic stability and length of action of that compound, the age, body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular

25

condition, and the host undergoing therapy.

The term "administered" includes delivery by viral or non-viral techniques. Viral delivery mechanisms include but are not limited to adenoviral vectors, adeno-associated viral (AAV) vectos, herpes viral vectors, retroviral vectors, lentiviral vectors, and baculoviral vectors. Non-viral delivery mechanisms include lipid mediated transfection, liposomes, immunoliposomes, lipofectin, cationic facial amphiphiles (CFAs) and combinations thereof. The routes for such delivery mechanisms include but are not limited to mucosal, nasal, oral, parenteral, gastrointestinal, topical, or sublingual routes.

The term "administered" includes but is not limited to delivery by a mucosal route, for example, as a nasal spray or aerosol for inhalation or as an ingestible solution; a parenteral route where delivery is by an injectable form, such as, for example, an intravenous, intramuscular or subcutaneous route.

GENERAL RECOMBINANT DNA METHODOLOGY TECHNIQUES

Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook et al., Molecular Cloning, A Laboratory Manual (1989) and Ausubel et al., Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. PCR is described in US-A-4683195, US-A-4800195 and US-A-4965188.

FIGURES

The present invention will now be described by way of example, in which reference shall be made to the following figures:

Figure 1 which presents a series of photographs and a graph;

Figure 2 which presents a photograph and a graph;

Figure 3 which presents a series of photographs and a series of graphs;

Figure 4 which presents graph;

Figure 5 which presents a series of photographs and a graph;

Figure 6 which presents graph;

Figure 7 which presents some bar charts;

Figure 8 which presents a graph;

Figure 9 which presents two graphs and two photographic images;

Figure 10 which presents a bar chart;

Figure 11 which presents a graph; and

Figure 12 which presents a bar chart.

The Figures will now be described in more detail.

Figure 1. Cannabinoid receptor agonism inhibits tremor in autoimmune encephalomyelitis¹. Mice with (a) hindlimb or (c) fore and hindlimb tremor both before (a & c) and after (b & d) treatment with 5mg/kg i.p. with *R(+)*-WIN 55,212. (e) Power spectra of hindlimb tremor recorded with the foot suspended above a strain gauge before (thick line) and after (thin line) 5mg/kg i.p. *R(+)*-WIN 55,212 injection. Snap-shot of raw record over 0.5 s in inset 1e.

Figure 2. Spasticity develops in autoimmune encephalomyelitis¹ (a) Spastic hindlimb showing full extension, including the digits. These were pressed against a strain gauge to measure the force required to bend the leg to full flexion. Raw recording in inset to 1a. (b) Increased resistance to flexion in post-relapse remission animals with spasticity (n=12 mice) compared with age-matched mice without evidence of spasticity (n = 5mice. * = P<0.001), or during active paralytic relapse episodes (n=6 mice. # = P<0.001).

Figure 3. Control of spasticity by the cannabinoid system. (a) Forces required to flex individual spastic hindlimbs against a strain gauge before and after injection with:

27

vehicle (0.1ml i.v. n=14); SR141716A (5mg/kg i.v. n=32); SR144528 (5kg/kg i.v. n=32); SR141716A and SR144528 (n=21 limbs); R(+)-WIN 55,212 (5mg/kg i.p. n=16) or S(-)-WIN 55,212 (5mg/kg i.p. n=19). (b-e) Cannabinoid receptor antagonism increased spasticity. (b) Before and after (c, e) SR141716A and SR144528 or (d) SR141716A administration. (c & d) Extension and crossing of limbs. (e) spastic tail. (f) Resistance to flexion forces 5min after SR141716A or SR144528 administration. 10min later mice were re-injected (5mg/kg i.v.) with either SR144528 (n=10), vehicle (n=15) or SR141716A (n = 18 limbs) and the resistance to flexion assessed 5min later. (g) Cannabinoid receptor agonism in spastic mice after either: R(+)-WIN 55,212 (n=16 limbs); Δ^9 -THC (n=18); methanandamide (n=23) or cannabidiol (n= 22). (*=P<0.001 compared to baseline). (h) Spasticity was (i) ameliorated by treatment with R(+)-WIN 55,212.

Figure 4. Treatment of spasticity in autoimmune encephalomyelitis¹ with non-CB₁ receptor agonists. Forces required to flex individual spastic hindlimbs against a strain gauge after injection i.v. with either low-dose methanandamide (n=9 limbs), JWH-133. (n=9) or palmitoylethanolamide (n=14). * P<0.05, **P<0.001 compared to baseline.

Figure 5. Spasticity is under tonic control of the endocannabinoid system. (a) The forces required to bend individual hindlimbs to full flexion against a strain gauge¹⁰¹ were assessed (>5mice/group) before and following injection i.v. with: rolipram (10mg/kg. n=12 limbs. RBI/Sigma, Poole, UK.), quinpyrole (1mg/kg. n=8limbs. RBI/Sigma, UK), AM404 (10mg/kg. n= 8 limbs) or AM374 (10mg/kg. n=7 limbs) *P<0.05 or **P<0.01 ***P<0.001 compared with baseline values. (b-c) Tail spasticity developed following (c) rolipram injection.

Figure 6. Spasticity is under tonic control of the endocannabinoid system at doses subthreshold for CB₁ receptor agonism. The forces required to bend individual hindlimbs to full flexion against a strain gauge¹ were assessed (>5mice/group) before and following injection i.v. with: AM404 (2.5mg/kg. N+12 limbs) or AM374 (1mg/kg. N=10 limbs) *P<0.05 or **P<0.01 ***P<0.001 compared with baseline values.

Figure 7. Endocannabinoid levels increase in spastic mice during CREA^E. Endocannabinoid levels were measured by isotope-dilution gas chromatography/mass spectroscopy from rapidly frozen brains and spinal cords removed from either normal

(N) or post-relapse remission animals either showing spasticity (S) or no clinical evidence of spasticity (NS), following the induction of CREAE (201).

Results represent mean \pm SD from 10-16 spinal cords/group (2 spinal cords pooled per sample) and 6-9 brains/group * P <0.05 ** P <0.01 compared to normal values by ANOVA.

Figure 8. *Spasticity is limited by endogenous cannabimimetic substances when exogenously administered.* The forces required to bend individual hind limbs to full flexion against a strain gauge were assessed (>6 mice/group) before and following injection i.v. with 10mg/kg anandamide (n = 11 limbs), 2-archidonoyl-glycerol (n=10 limbs) and as previously reported (3) palmitoylethanolamide (n = 14 limbs) * = P < 0.05, ** = P < 0.01, *** = P < 0.001 compared with baseline values by ANOVA.

Figure 9. *Spasticity is under tonic control of the endocannabinoid system.* The forces required to bend individual hind limbs to full flexion against a strain gauge were assessed (>5mice/group) before and following injection i.v. with: (A) rolipram (10 mg/kg. n=12 limbs), quinpirole (1 mg/kg. n=8 limbs.), AM404 (10 mg/kg. n= 8 limbs) or AM374 (10 mg kg. n=7 limbs), (B) AM404 (2.5 mg/kg, n= 10 limbs), VDM11 (10 mg/kg, n= 11 limbs), AM374 (1 mg/kg, n=15 limbs) or AM374 (1 mg/kg, n = 12 limbs) 20 min after injection i.v. with 5 mg/kg of both SR141617A and SR144465 (SR1&2). * = P < 0.05, ** = P < 0.01, *** = P < 0.001 compared with baseline values by ANOVA. (C-D) Tail spasticity developed following (D) rolipram.

Figure 10 - *Spasticity is limited by Arvanil.* Following the development of spasticity occurring after CREAE induction, the forces required to bend individual hind limbs to full flexion against a strain gauge were assessed (>6 mice/group) before and following injection i.v. with arvanil, linvanil, capsaicin and methanandamide at the doses indicated. (A) The results represent the mean \pm SEM percentage change in resistance to flexion 30 minutes after injection compared to baseline levels (0%) at 0 min. ** = P < 0.01, *** = P < 0.001 compared with baseline values by ANOVA.

Figure 11 - *Spasticity is limited by Arvanil and vanilloid recetpor agonists* Following the development of spasticity occurring after CREAE induction, the forces required to

bend individual hind limbs to full flexion against a strain gauge were assessed (>6 mice/group) before and following injection i.v. with arvanil 0.1mg/kg, capsaizepine (50mg/kg), capsaicin (0.1mg/kg) or capsaicin injected animals that had been pretreated (-10min) with capsaizeopine. The results represent the group mean \pm SEM. resistance to flexion 30 minutes after injection compared to baseline levels $** = P < 0.01$, $*** = P < 0.001$ compared with baseline values by ANOVA.

Figure 12 - Spasticity is limited by VR-1 and CB1 receptor-mediated effects. Following the development of spasticity occurring after CREAE induction, the forces required to bend individual hind limbs to full flexion against a strain gauge were assessed (>6 mice/group) before and following injection i.v. with capsaizepine (50mg/kg iv), capsaicin (0.1mg iv) or R(+)WIN-55,212 (5mg.kg ip) The results represent the mean \pm SEM percentage change in resistance to flexion 30 minutes after injection compared to baseline levels (0%) at 0 min. In some instance animals were pretreated (-20min) previously with capsaizepine (50mg/kg i.v.) a combination of SR141716A and SR144528 (SR1 & SR-2 5mg/kg iv) or both. $* = P < 0.05$, $** = P < 0.01$, $*** = P < 0.001$ compared with baseline values by ANOVA.

EXAMPLES

EXAMPLE 1

METHOD

Induction of CREAE. Biozzi ABH mice, bred at the Institute of Ophthalmology, were injected with 1mg of mouse spinal cord homogenate emulsified in Freund's complete adjuvant on day 0 and 7¹. Animals injected for CREAE, prior to the onset of acute phase CREAE¹ (usually occurring between day 15-20 post-inoculation (p.i.)) were used as normal CREAE controls. Paralysed CREAE animals were selected during the acute phase or first relapse (typically occurring on days 35-45 p.i.), and remission animals used for the assessment of tremor and spasticity were used following the second/third relapse (days 50-80p.i.).

30

Chemicals. *R*(+)-WIN 55,212, *S*(-)-WIN 55,212, Δ^9 -THC, methanandamide and cannabidiol were purchased from RBI/Sigma (Poole, UK). Palmitoylethanolamide was purchased from Tocris Cookson Ltd (Bristol, UK). SR141716A¹⁵ and SR144528¹⁶ were supplied by Dr. M. Mossé and Dr. F. Barth (Sanofi Research, Montpellier, France). JWH-133 (3-(1'-1'-dimethylbutyl)-1-deoxy- Δ^8 -THC) was synthesised as described previously¹⁹. All compounds were dissolved at 0.5mg/ml in ethanol containing 1mg/ml Tween 80 (Sigma). The ethanol was removed by vacuum drying, then samples were reconstituted with phosphate buffered saline to a concentration of 2mg/ml. Similar preparations without active drug were used as vehicle controls. Suspensions (0.1ml) were injected either i.v. or i.p. following CREAE induction.

Assessment of Clinical Signs. Spasticity and tremor was assessed initially by blinded analysis of video recordings. Digital images were sampled from video at 0.04s. Signs of tail spasticity (flicking and curling) were assessed visually as being either present or absent. Spasticity was confirmed by assessing limb spasticity against a small purpose-built strain-gauge. Limbs of animals without clinical evidence of spasticity (propensity to fully extend the limb following tension to the leg) or the propensity to cross were not examined in drug studies. The analogue signal was amplified and digitally converted using an Amplicon card. (Brighton, UK) This was captured using dacquire V10 software (D. Buckwell, MRC HMBU, Institute of Neurology) and analysed using Spike 2 software (Cambridge Electronic Design, UK). The hindlimbs were fully extended twice then moved to full flexion against the strain gauge. Each hindlimb was assessed individually by a blinded operator and 4-8 individual readings made per limb and the mean taken. Tremor frequency and severity were also recorded by holding the limb ~5mm above the strain gauge. Tremor lead to knocking of the strain gauge by the foot. The strain gauge output was notch filtered at 50 Hz. The device had a resonance frequency of 95 Hz. The frequency of limb tremor was also confirmed using a light-weight unidirectional accelerometer (EGA XT-50, Entrain, UK) mounted over the foot.

Statistical Analysis. Results are expressed as mean of individual feet/animals \pm SEM per group. The data were assessed using either a *t* test, paired *t* test for flexion data or nonparametric Mann Whitney U test using SigmaStat 2.0 software (Jandel Corp, San Rafael, CA, USA).

RESULTS AND DISCUSSION

It is well established that during CREAE repeated neurological insults occur, which are associated with increasing primary demyelination and axonal loss in the central nervous system (CNS)¹. However it was evident also that CREAE animals can develop additional clinical signs including, unilateral or bilateral, fore and hindlimb tremor (Fig 1) and hindlimb spasticity (Fig 2). These accumulate with disease duration and activity. Tremor was associated with voluntary limb movements, but in more severe cases was persistent, at a frequency of ~40Hz (Fig 1e). While considerably faster than encountered in humans (~6Hz), this frequency is consistent with electromyography of tremor in mutant spastic (*Glr^{Spa}*) mice¹¹. These animals develop episodes of rapid tremor and rigidity of the limb and trunk muscles¹². However unlike the *Spastic* mouse, spasticity in CREAE mice need not be triggered by sudden disturbance¹². The effects of cannabis are mediated through the CB₁ and CB₂ and CB₂-like receptors^{13,14}. CB₁ is predominant in the CNS and is the major target for psychoactivity, but is also expressed at lower levels in many peripheral tissues. The CB₂ receptor is expressed in high levels on leucocytes, but there is also evidence for limited CB₂ receptor expression in mouse brain^{13,14}. The administration of a full CB₁ and CB₂ agonist, *R*(+)-WIN 55,212⁸ to post-relapse remission mice resulted in a rapid (within 1-10min) amelioration of the frequency and amplitude of tremor in both the fore and hindlimbs of CREAE mice (Fig 1). This was visually evident at 5mg/kg (Fig 1a-d. n= 10/10) and 1mg/kg i.p. (n= 6/6). In addition Δ⁹-THC (10mg/kg i.v.) also ameliorated this response (n=5/5). Tremor returned within hours after treatment. As Δ⁹-THC was observed to be relatively ineffective via the i.p. route, as seen in other studies¹⁰, all subsequent compounds were injected via the i.v. route. Furthermore as Δ⁹-THC is a partial CB₁ agonist but provides more limited CB₂ agonist activity, these results suggest that the effect on tremor is mainly mediated via the brain CB₁ receptor⁸.

Pretreatment (10min) of animals with 5mg/kg i.v. of both selective CB₁ (SR141716A)¹⁵ and CB₂ (SR144528)¹⁶ receptor antagonists prevented the capacity of 5 mg/kg i.p. *R*(+)-WIN 55,212-2 to inhibit tremor (n=5/5). However animals with residual paresis and mild spasticity became significantly more spastic following such CB receptor antagonism (Fig 3). This was associated with uncontrolled leg crossing (Fig 3c & d) and severe tail spasms. These showed gross curling which is atypical of post-remission animals where the tail generally hangs limply (Fig 3e). Animals also

32

show hindlimb extension (Fig 3c), including a significant ($P < 0.0001$) increase in resistance to flexion (Fig 3a & f). This was not observed in vehicle-treated controls (Fig 3a). These signs were also not evident in similarly-injected normal mice ($n=0/5$) or normal appearing pre-acute EAE animals (hindlimb resistance to flexion $0.159 \pm 0.013N$ vs. $0.206 \pm 0.022N$ in treated mice. $n=12$ limbs. $P > 0.05$) and in animals with paresis/paralysis without evidence of spasticity ($n=0/5$ treated with SR141716A and SR144528. $n=0/4$ treated with SR141716A or SR144528 alone). When mildly spastic animals without tremor were injected with 5mg/kg i.v. CB_1 antagonist not only did significant hindlimb ($P < 0.001$. Figure 3a) and tail spasticity ($n=18/18$. $P < 0.001$) develop compared with vehicle treated controls ($n = 0/6$.), but also forelimb tremor became evident in 3/10 mice. Thus implicating a role of CB_1 in the control of tremor. Following injection of 5mg/kg i.v. CB_2 antagonist some animals ($n=10/14$) appeared to demonstrate a mild increase in the level of tail spasticity ($P < 0.02$) and showed a small but significant ($P < 0.05$) increase in resistance to hindlimb flexion (Fig 3a). However, when the CB_2 antagonist was injected into animals previously made more spastic ($P < 0.01$) by CB_1 antagonism, spasticity increased significantly ($P < 0.001$) compared to animals treated with SR141716A alone, whereas this resolved in vehicle treated animals. This suggests that both CB receptors may control spasticity (Fig 3f). However, it is possible that the effects of SR144528 could be mediated by CB_2 -like (rather than CB_2) receptors as previously proposed¹⁷, or that at the dose used, SR144528 may have produced additional CB_1 antagonism because it has some limited capacity to bind to CB_1 ⁸. These observations may be an indication of ongoing release of endogenous cannabinoid receptor agonists such as anandamide and 2-arachidonylglycerol which are present within the brain and exhibit neurotransmitter function¹⁸. Alternatively, or in addition, they may reflect the presence of precoupled, constitutively active cannabinoid receptors, there being evidence that SR141716A and SR144528 are both inverse agonists that are capable of producing inverse cannabimimetic effects by reducing the proportion of cannabinoid receptors that exist in a precoupled state^{8,15,16}. In comparison to some studies antagonising the effect of exogenous agonists¹⁷, the action of the antagonists seen here were relatively short-lived (Fig 3f). This may reflect that the animals were attempting to compensate for the antagonist effect, and would be consistent with tonic control of the endogenous cannabinoid system. Importantly, these data provide compelling evidence that CB receptors are involved in the control of spasticity within an environment of existing neurological damage, and that exogenous agonism may provide benefit.

Indeed in mice with significant spasticity, 5mg/kg i.p. *R*(+)-WIN 55,212 reduced severity both visually (n=7/7. Fig 3g & h) and following ($P<0.001$) assessment of resistance to hindlimb flexion (Fig 3a & i). This was also evident with 2.5 mg/kg i.p. *R*(+)-WIN 55,212 (Resistance of flexion of both limbs being reduced ($P<0.05$) from $0.384 \pm 0.096\text{N}$ to $0.276 \pm 0.063\text{N}$ n=7. $P<0.05$). Similar treatment with 5mg/kg i.p. of the inactive enantiomer *S*(-)-WIN 55,212 failed to significantly affect the spastic response (Fig 3a). In contrast 10mg/kg i.v. Δ^9 -THC and 5mg/kg i.v. methanandamide (CB_1 -selective. K_i for $\text{CB}_1 = \sim 20\text{nM}$ and K_i for $\text{CB}_2 = \sim 815\text{nM}$)⁸ induced a significant ($P<0.01$) amelioration in spasticity (Fig 3i). This, coupled with the observations using SR141716A may suggest further that CB_1 is a major target for control of spasticity. Currently there are no compounds which are totally CB_1 or CB_2 receptor specific, but the lack of effect following 10mg/kg i.v. cannabidiol (major non-psychoactive component of cannabis. K_i for $\text{CB}_1 = 4350\text{nM}$)⁸ suggested a subthreshold dose for CB_1 stimulation for treatment of spasticity. Using the CB_2 - selective agonist JWH-133¹⁹ (1.5mg/kg. i.v. K_i for $\text{CB}_1 = \sim 680\text{nM}$ and K_i for $\text{CB}_2 = \sim 3\text{nM}$ ^{8,19}) spasticity was reduced both 10min ($P<0.05$) and 30min ($P<0.001$) following injection at a time when 0.05mg/kg i.v. (dose selected to exhibit similar CB_1 activity to JWH-133) methanandamide was not active (Fig 4). While it is possible that "sedative" effects may have contributed (though CB_1 -receptors) to cannabinoid-mediated effects in these assays, there was no hypothermia, indicative of "sedation" following JWH-133 administration ($37.1 \pm 0.2^\circ\text{C}$ (baseline), $37.2 \pm 0.4^\circ\text{C}$ (10min) $37.1 \pm 0.2^\circ\text{C}$ (30min)). That non- CB_1 receptors may also control spasticity is further supported by transient inhibition of spasticity with the endocannabinoid palmitoylethanolamide (Fig 4). This compound has no significant affinity for CB_1 but may have activity for CB_2 -like receptors⁸. The involvement of non- CB_1 receptors may be definitively resolved through the use of CB receptor subtype-specific compounds or CB receptor deficient mice.

Spasticity in patients with MS can be very difficult to control despite the use of oral baclofen, dantrolene, diazepam and tizanidine, continuous intrathecal baclofen infusion and selective injection of botulinum toxin²⁰. There is a need for more effective oral or systemic anti-spasticity agents. The hydrophobic nature of the cannabinoids provides for rapid access to the CNS. While the effects of chronic administration and dose-dependency of CB receptor agonists on experimental spasticity remain to be investigated further, the data presented here provide evidence

for the rational assessment of cannabinoid derivatives in the control of spasticity and tremor in MS, in placebo-controlled trials. The observation that CB₁ appears to be the major therapeutic target, suggests that it may be difficult to dissociate full benefit from undesirable psychoactive elements using Δ^9 -THC or cannabis, and would be consistent with the unpleasant side effects experienced by some patients at the doses required for potential therapy by existing cannabinoids³. Use of selective CB₂ agonists may provide some symptomatic benefit without significant psychoactive effects. Furthermore it may be possible to up-regulate endogenously-produced cannabinoids¹⁸ to mediate therapeutic benefit. This CREAE model provides an avenue to evaluate and control, the pathophysiology of spasticity within a chronic inflammatory environment relevant to the control of MS.

Hence, cannabinoids control spasticity and tremor in a multiple sclerosis model.

EXAMPLE 2

Anandamide was the first endogenous cannabinoid receptor ligand identified, and biosynthetic pathways have been described⁵. Recently anandamide was found to regulate dopamine-mediated locomotor activity, and D₂ receptor agonism with quinpyrole can induce a limited release of anandamide, and augment the effects of exogenous anandamide⁶.

However, no amelioration of spasticity was evident following administration of quinpyrole alone (1mg/kg i.p. (Fig 5), and 10mg/kg i.p. (n=8 limbs). RBI/Sigma, Poole, UK). Following release into the extracellular space, anandamide is actively removed into the cytosol through specific carrier proteins^{103, 105}. Anandamide also undergoes hydrolysis to arachadonic acid and ethanolamine, via the action of fatty acid amino hydrolases (FAAH)^{104, 105}. Inhibition of these activities therefore serves as an alternative way to increase availability of endocannabinoids, in order to limit spasticity.

We have found that injection (10mg/kg i.v.) of the competitive reuptake inhibitor, AM404¹⁰³ or a selective FAAH inhibitor; AM374¹⁰⁴ ameliorated spasticity (Fig 5a). It appeared that no additional benefit was evident by using both these reagents in combination (From a baseline of $0.289 \pm 0.026N$ (n=18) such treatment significantly reduced spasticity at 10min ($0.254 \pm 0.034N$, $P<0.05$), 30min ($0.181 \pm 0.029N$, $P<0.001$), and 1 hour ($0.200 \pm 0.028N$, $P<0.001$)). While this further supports the role of the endocannabinoids in the control of spasticity, both of these compounds have limited affinity for cannabinoid receptors^{103, 104} and thus could exhibit some receptor agonism. However there is no evidence for cannabinometric effects of AM404¹⁰³ or AM374 (no significant temperature change following 10mg/kg i.v.) at the doses used here *in vivo*. Furthermore significant ($P<0.001$) anti-spasticity effects were also evident using doses AM404 (2.5mg/kg) and AM374 (1mg/kg) likely to be subthreshold for CB₁ agonist control of spasticity¹⁰¹. Therefore cannabinoid receptor agonism is unlikely to account for the inhibitory activity. Importantly however, was the observation that reduction of endocannabinoids levels increased the severity of signs. (Fig 5a-c). Anandamide is not stored but is "produced on demand" from preformed membrane phospholipid precursors by phosphodiesterase (PDE) catalytic activity¹⁰⁶. A number of PDE (IV) inhibitors, such as rolipram, have been shown to

have anti-inflammatory effects and can inhibit the immunological processes that drive the development of clinical deficit in rodent and primate EAE^{107, 108}. In contrast, injection (10mg/kg i.v.) of rolipram (RBI/Sigma, Poole UK) induced a transient increase in spasticity shown by an increase ($P < 0.001$) in resistance to hindlimb flexion (Fig 5a) and tail spasticity (Fig 5b,c. $n = 15/15$. $P < 0.001$ compared with normal animals $n = 0/5$. Indeed this appeared to transiently (1hour) sedate the ABH mice). Furthermore limb tremor became evident in some mice ($n = 6/15$). The increased activity seen following rolipram treatment was transient (Fig 5a), consistent with that seen previously with cannabinoid receptor antagonists (SR141716A and SR144528.¹⁰¹), and was not evident in normal animals. This again suggests that compensatory mechanisms are rapidly activated, but demonstrates clearly that the endocannabinoid system exhibits tonic control of spasticity.

As a consequence of neurological damage in MS and CR-EAE the equilibrium of the cannabinoid system appears to be altered in an attempt to restore neuromuscular control, but it does not adequately prevent the occurrence of spasticity. However it is possible achieve benefit through supplementary cannabinoid receptor agonism, as demonstrated in CR-EAE¹⁰¹ and perceived by MS patients¹⁰⁹ but also by cannabinoid reuptake and breakdown inhibitors. These latter compounds may limit some of the undesirable psychotropic activities associated with the use of CB₁ receptor agonists. Parallels can already be seen in the treatment of depression where serotonin reuptake inhibitors appear clinically preferable to serotonin receptor agonists¹¹⁰. This CREAE model provides a way to dissect these mechanisms, but also to way to examine the effects of possible combinations of reagents that will lead to a sustained therapeutic effect in multiple sclerosis and other neuromuscular disorders.

Hence, endocannabinoids limit spasticity in a multiple sclerosis model.

EXAMPLE 3

Introduction

As indicated above, Multiple sclerosis (MS) is a chronic, demyelinating disease of the central nervous system (CNS), where individuals accumulate neurological damage

and paresis. In addition, troublesome signs often develop, such as pain, spasticity and tremor that are difficult to treat. This has prompted some patients to find alternative medicines, and to perceive benefit from cannabis use (201). The effects of cannabis and cannabinoids are mediated through cannabinoid receptors (CB) of the type 1 (CB₁), expressed mainly in the CNS but also in peripheral tissues, and type 2 (CB₂), expressed almost uniquely in immune cells (202). Recently, we have demonstrated that exogenous agonists of CB receptors, in particular CB₁, can inhibit spasticity in the Biozzi ABH mouse chronic relapsing experimental allergic encephalomyelitis (CREAE) model of MS (203). This autoimmune demyelinating disease is actively induced by sensitization to CNS myelin (204). Systemic agonists will not discriminate between CB receptors located in centers associated with control of pain, motor or cognitive functions, and therefore, may induce unwanted psychoactive effects that will limit medical alleviation of pain/motor deficits. More importantly we demonstrated that CB antagonism transiently exacerbated spasticity (203). Whilst inverse agonism of antagonists (202) may explain the latter observation, alternatively this suggested that endogenous CB ligands are limiting the spasticity that occurs during CREAE. This would predict that spasticity would be associated with changes in the equilibrium of the endocannabinoid system (5), which could be manipulated for therapeutic benefit by using inhibitors of endocannabinoid degradation.

METHODS

CREAE Induction. Biozzi ABH mice were from stock bred at the Institute of Ophthalmology, UCL London or were purchased from Harlan Olac (Bicester, UK). CREAE was induced following subcutaneous injection of 1mg of syngeneic spinal cord homogenate emulsified in Freund's complete adjuvant (Difco, Poole, UK) on day 0 and day 7 as described previously (4). Animals developed a relapsing-remitting disease progression and between 60-80 days post-inoculation developed evidence of spasticity (incidence 50-60%) (203). Similarly treated CREAE animals that had not yet demonstrated tremor, hindlimb or tail spasticity were used as non-spastic controls.

Assessment of Endocannabinoid levels. The spinal cords were rapidly expelled from the cervical spinal column using hydrostatic pressure applied through a 19 gauge needle inserted into the lumbar column via a phosphate buffered saline-filled syringe. Brains were dissected from the cranium. All tissues were frozen in liquid N₂ within 60s from death (206). AEA, PEA and 2-AG levels in lipid extracts from mouse brain and spinal cord were assessed using isotope-dilution gas chromatography/mass spectroscopy essentially as described previously (207, 208). Results were compared by oneway analysis of variance (ANOVA) incorporating a Bonferroni *t* test. Statistical analysis was performed using SigmaStat V2 software (SPSS Inc., USA).

Assessment and Modulation of Spasticity. Quinpirole, rolipram and R(+)WIN-55,212 were purchased from RBI/Sigma (Poole, UK). Anandamide, PEA and 2-AG were purchased from Cayman chemical (Ann Harbor Mi, USA). AM404 (209), AM374 (210) and VDM11 (211) were synthesized as previously described. The CB receptor antagonists SR141617A and SR144465 (202) were supplied by Sanofi Research (Montpellier, France). Ethanolic solutions were evaporated under vacuum and dissolved in PBS:tween 80 (Sigma,UK) (203) to be administered as a single intraperitoneal or intravenous tail injection. The resistance to flexion of individual hind limbs was measured against a strain gauge (5-8 readings per time point) as described previously (203). The results were expressed as a mean \pm SEM per group, and the data was analyzed using repeated measures ANOVA incorporating a pairwise Tukey post-hoc test.

RESULTS AND DISCUSSION

Endocannabinoid levels in spastic mice. At baseline in normal ABH mice (Fig.7), whole brains and spinal cords contained similar levels of the endocannabinoids arachidonylethanolamide (AEA/anandamide, ~29-33 pmol/g) and 2-arachidonoyl glycerol (2-AG, ~5-7 nmol/g) and the non-CB receptor binding, cannabimimetic metabolite (202), palmitoylethanolamide (PEA, ~220-240 pmol/g), as found previously in rat CNS tissue (207, 208). These levels were not significantly changed in non-spastic CREA remission animals (Fig.7), despite the fact that these animals had experienced 2-3 paralytic episodes and would contain demyelinated lesions and

axonal loss in the spinal cord (4). However in comparison to normal animals, endocannabinoids were present in significantly ($P < 0.05$) elevated amounts in the brain of spastic mice (Fig 7). Although brain levels were relatively unchanged for AEA compared ($P > 0.05$) with non-spastic mice, there was a modest increase of AEA (5) ($P < 0.05$) in spastic brains compared with levels in normal brains. However, there was a marked increase (~200%) of AEA ($P < 0.01$) and PEA ($P < 0.05$) within the spinal cord of spastic mice (Fig 7). This is the site of major pathological change that occurs during CREA in ABH mice (204, 206). The brain during CREA is relatively unaffected but animals do develop lesions within the cerebellum (204), an area 10 implicated in the control of tone. Due to the low levels of AEA (pmol/g), subtle changes in the amounts of this metabolite may not be as readily detected in whole brain, as opposed to isolated brain regions (208). On the other hand, 2-AG is found at 200-800 times higher levels than AEA and here an increase (~70%) in 2-AG levels was readily found in brain and spinal cords ($P < 0.05$) of spastic animals compared 15 with non-spastic controls (Fig 7). While this finding may suggest that 2-AG could serve as a more-easily detectable indicator of endocannabinoid activity during spastic disease, 2-AG has not been detected in micro-dialysates from rat striatum (212), or in human cerebrospinal fluid (CSF) (213), despite its relative abundance in CNS tissues (205, 207, 208). This is possibly due to tissue compartmentation of 2-AG (213), to its 20 very limited release from neuronal cells (214) or to its rapid esterification into phospholipid membranes and hydrolysis (215, 216). This study indicates that the endocannabinoids are up-regulated locally in areas of CREA-induced damage. Therefore, clinical parameters, i.e. spasticity vs. non-spasticity and lesion load/topography, need to be considered when samples from MS patients are examined 25 for human confirmation of these animal data. As post-mortem artefactual increases of endocannabinoids may mask changes in human CNS tissue (217), studies in humans will rely on examination of the CSF.

Endocannabinoids inhibit spasticity. The lack of changes of endocannabinoid levels 30 between normal and non-spastic mice and elevations of endocannabinoids in spastic mice may provide an explanation to previous observations obtained using CB receptor antagonists (203). In this previous study it was shown that exogenously administered SR141617A and SR144465, two antagonists selective for CB₁ and CB₂ receptors,

failed to affect resistance to flexion of hind limbs (muscle tone) in normal and non-spastic CREAE mice, in contrast to spastic animals, which showed a transient elevation in resistance to flexion of the hind limbs with both antagonists (203). On the basis of the results described above, these observations can now be explained by hypothesizing that the two antagonists block the action of disease-limiting endocannabinoids, whose levels are elevated during spastic disease. Although it is speculated that endocannabinoid levels may increase in an attempt to compensate for the spastic defect, it is possible that endocannabinoids, or other unrelated fatty acid amides, could be transiently elevated as a mere consequence of spasticity, such as increased motor activity (212, 220), or tissue damage (205), rather than exerting a compensatory effect on this sign. Whilst exogenous administration of methanandamide (AM356), an enzymatically stable AEA analogue (221), can limit experimental spasticity (203), in order to suggest a cause/effect relationship between endocannabinoids and inhibition of spasticity, the effect of exogenous AEA, 2-AG and PEA was investigated here. It was found that all three substances had the capacity to significantly ($P < 0.01$) ameliorate spasticity, although they had differing efficacy profiles (Fig 8). Whilst AEA and PEA maximally inhibited spasticity within 10-30 min, exogenous 2-AG induced inhibition with a relatively slower onset. (10 mg/kg i.v. Fig 2, and 1 mg/kg i.v. $n=13$ limbs, data not shown). This was somewhat surprising since endocannabinoids are susceptible to rapid inactivation through enzymatic hydrolysis (5, 15). Although different cannabinoids have different pharmacokinetics (203), this observation may also suggest that 2-AG is not directly mediating the inhibition, and that this could result in part from the actions of other bioactive metabolite(s). For example, 2-AG may also act by slowly inhibiting the degradation of endogenous AEA thereby increasing its levels (15, see below). Alternatively, 2-AG activates CB_2 receptors more efficaciously than AEA, and this different mechanism of action may also explain the different profile of spasticity inhibition observed here for the two endocannabinoids. As for PEA, this endogenous compound does not exhibit CB_1 or CB_2 agonist activity (202) but may also be capable of enhancing endocannabinoid actions, as shown in suppression of signs of hyperalgesia (222) and inhibition of breast cancer cell proliferation (223), through not fully understood effects (223). Similar to AEA, the levels of this metabolite were found here to be raised in CREAE mice spinal cord (Fig. 7) and to transiently ameliorate spasticity

(203. Fig 8). Thus, PEA appears to be linked somewhere to the endocannabinoid system even though it is not an endocannabinoid itself.

Inhibition of Endocannabinoid degradation in the control of Spasticity. Having established that the endocannabinoids are upregulated during spastic CREAE, a causal relationship to the anti-spastic effect was sought through manipulation of endocannabinoid levels and action *in vivo* (Fig 8). AEA was the first endocannabinoid described and its biosynthetic/degradation pathways have been best described (5, 24). Since exogenous applied naturally-occurring cannabimimetic metabolites, in particular AEA, can limit spasticity (Fig 202), augmenting the levels of endogenous AEA might have a therapeutic effect. One route would be to induce its synthesis. AEA has been reported to regulate dopamine-mediated locomotor activity, whereas D₂ receptor agonism with quinpirole induced the release of AEA, and augmented the dopamine inhibiting effect of exogenous AEA in mice (212). However, no amelioration of spasticity was evident following administration of quinpirole alone (1 mg/kg i.v. Fig 8) and 10 mg/kg i.p. (data not shown, n=8 limbs). This suggests that such stimulation either fails to induce sufficient AEA to control spasticity, or that AEA production was altered in an anatomical site away from the spastic lesion (220).

20

Blockade of degradation with specific inhibitors may serve as an alternative means of increasing the bio-availability of the endocannabinoids. Furthermore this strategy would provide some selectivity as these inhibitors would particularly target areas where elevated levels of the endocannabinoids are being produced and utilized. AEA, and possibly 2-AG, are actively removed from the extracellular space into the cytosol through specific mechanisms (9, 15) and undergo breakdown by fatty acid amide hydrolase (FAAH) (5, 15, 24, 25). We found that spasticity could be ameliorated by injection (10 mg/kg i.v.) of either the competitive reuptake inhibitor AM404 (209), or the selective FAAH inhibitor, AM374 (210), both of which have been shown to enhance AEA neuromodulatory actions (209, 226) (Fig 8a). No additive effect was evident using a combination of both reagents (n=18, data not shown). These compounds have very low affinity for cannabinoid receptors (209, 210), and have never been shown to behave as CB agonists (227). In fact, there was no evidence for

30

cannabimimetic effects (hypothermia) of AM404 or AM374 at the doses used here *in vivo* (data not shown). Furthermore, significant ($P < 0.001$) anti-spasticity effects were also evident using doses of AM404 (2.5 mg/kg) and AM374 (1 mg/kg) likely to be sub-threshold for CB₁ agonist control of spasticity (Fig 8b. (203)). These inhibitory effects had a rapid onset before a slow return of CREA signs over the next few hours (Fig 202), and were comparable to those obtained following effective CB receptor agonism (203). Both AEA and AM404 may also behave as vanilloid receptor (VR1) agonists (218, 219) and other VR1 agonists have been found to reduce bladder hyper-reactivity in multiple sclerosis MS (228). Whilst the role of VR1, if any, in control of spasticity has yet to be demonstrated, a similar inhibition ($P < 0.001$) of spasticity (Fig 8b) by the extremely selective anandamide transporter inhibitor VDM11 (10 mg/kg i.v.), which has essentially no CB or VR-1 agonist activity (211), further supports the hypothesis that endocannabinoids mediate control of spasticity via CB receptors.

Here we gained indirect evidence that at least AM374 is acting via this mechanism. In fact, the anti-spastic effect of AM374 (1 mg/kg i.v.) was blocked by cannabinoid receptor antagonists (SR141716A and SR144465, both 5 mg/kg i.v.) administered 20 min prior to AM374 (Fig 3b). Under the same conditions the two antagonists were shown to counteract the anti-spasticity effects of the CB receptor agonist *R*(+)-WIN-55, 212 [5 mg/kg i.p., change in resistance to flexion at 30 min $+7.0 \pm 18.1\%$ ($P > 0.05$ $n = 11$ limbs) compared to baseline in antagonist-pretreated animals, as opposed to $-35.5 \pm 19.7\%$ ($P < 0.05$ $n = 16$ limbs) in vehicle-pretreated mice receiving *R*(+)-WIN-55, 212 alone]. These findings suggest that the inhibitory effect on spasticity by AM374, which does not directly activate CB receptors (210), is due to enhancement of endocannabinoid levels and subsequent stimulation of CB receptors.

Inhibition of Endocannabinoid signalling exacerbates spasticity. As CB receptor antagonism very transiently exacerbates spasticity (208), we studied the effect on spasticity of inhibiting CB receptor signaling (Fig 8a). CB receptors are negatively coupled through G_{i/o} proteins to adenylate cyclase, and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine inhibits receptor agonism (229). Rolipram, a selective inhibitor of cAMP-selective phosphodiesterase IV, has anti-inflammatory

effects and inhibits the immunological processes that drive the development of EAE (230). However, in agreement with its possible counteraction of endocannabinoid-induced inhibition of cAMP levels, rolipram (10 mg/kg i.v.) induced a transient increase ($P < 0.001$) in limb (Fig 3a) and tail spasticity ($n = 15/15$, $P < 0.001$ compared with normal animals $n = 0/5$. Fig 3c-d). Furthermore, limb tremor became evident in some mice ($n = 6/15$). The exacerbation was not evident in normal animals, where rolipram appeared to have a sedative effect, and was very transient (Fig 3a), consistent with that observed previously with CB antagonists (203). This finding again suggests that compensatory mechanisms are rapidly activated after exacerbation of spasticity in CREAE mice, and substantiates further the involvement of the endocannabinoid system in the tonic down-regulation of this sign.

Conclusions. This study provides strong evidence for a role of endocannabinoids in the control of spasticity. The equilibrium of the endocannabinoid system appears to be significantly altered during spastic events in CREAE, possibly in response to abnormal neuronal signaling and/or neurodegenerative effects in damaged nerves. However, this phenomenon does not control spasticity as adequately as it may be possible by administering exogenous CB agonists (203) - including drugs based on endocannabinoids that have been reported to have very low potential for physical dependence (231) - or by manipulating endocannabinoid endogenous levels. This manipulation may minimize some of the undesirable psychoactive effects associated with CB₁ agonism and may have implications for symptom control in MS, and other neuromuscular disease conditions. Parallels can already be seen in the treatment of depression where serotonin reuptake inhibitors are clinically preferable to receptor agonists. The finding of increased amounts of endocannabinoids in these damaged tissues may open even wider horizons for therapeutic intervention in MS with little psychotropic side-effects.

EXAMPLE 4**ARVANIL INDUCED INHIBITION OF SPASTICITY****BACKGROUND**

5 Pain, bladder instability and spasticity are common symptoms in multiple sclerosis (MS), and other conditions such as spinal injury, for which there are a paucity of effective therapies. Novel therapies for the alleviation of these symptoms, an area of therapeutic need, may be based on recent developments in the pharmacology of
10 cannabinoids (CB) and vanilloids.

The pungent ingredient of chilli peppers, capsaicin, exerts its effects via the noxious-heat gated ion channel vanilloid receptor 1 (VR1), which is selectively expressed on primary afferent nociceptive neurones (1,2). Persistent activation of VR1 results in
15 de-sensitisation of nociceptors and thus analgesia. There is laboratory evidence that the bladder hyper-reflexia which develops following inflammation or spinal cord disease is attenuated by capsaicin. Furthermore, clinical intra-vesical therapy with capsaicin has enjoyed some success in alleviating bladder symptoms in patients with both MS or spinal cord injury, despite the invasive route of application and the
20 stimulant nature of capsaicin. In addition there is clinical evidence that topical capsaicin therapy is effective for the treatment of neuropathic pain (e.g. post herpetic neuralgia or painful diabetic neuropathy), but again the stimulant side-effects limit its usefulness (3). However non-stimulating systemically administered capsaicin analogues represent a significant therapeutic advance in this field (4). In animal
25 models, VR1 receptor agonists ameliorate pain and bladder instability, and can be also attenuated by cannabinoids. So far two CB receptors (CB₁ and CB₂) have been cloned. The CB₁ receptor is expressed by neurones of the central and peripheral nervous system and activation of this receptor and plays a role in down regulating the nerve growth factor (NGF) component of inflammatory hyper-algesia and bladder
30 hyper-reflexia (5).

Endogenous ligands at CB receptors have been identified, including the prototypical ligand anandamide (6) and their synthesis, and degradation and inactivation pathways described (6). CBs are effective in relieving neuropathic pain, in the latter
35 there may be a therapeutic advantage of CBs over opioids (7) and of bladder hyper-

45

- reflexia (8-9). Recently it has reported that anandamide exerts agonist activity at both the VR1 and CB1 receptors (10-11). In addition, synthetic vanilloid receptor agonists, such as olvanil, can interact with CB1 cannabinoid receptors and the anandamide re-uptake transporter (AT) (12). Metabolically stable anandamide analogues that can be administered systemically in animal models have been synthesized (13) and one of these analogues (arvanil [D5,8,11,14-cis C20:4 n-6 N-acyl-vanillyl-amide]) behaves as a "hybrid" ligand at both CB₁ (but is non CB₂ binding) and VR1 receptors and arvanil also acts as a potent inhibitor of the AMT.
- 10 It has been demonstrated that CB receptor agonists, largely mediated via CB₁, can inhibit spasticity in an MS-like animal model (14). This will serve to augment natural endocannabinoid-induced inhibition of this sign (15). Furthermore enhancement of the endocannabinoid levels through inhibition of endocannabinoid degradation (6, 15), also leads to amelioration of spasticity (15). Thus an agent that targets the VR-1 and CB systems may have the potential to provide relief of a range of diverse symptoms. Arvanil is a systemically bio-available agents in this category could provide a significant therapeutic advance for the treatment of pain, spasticity and of bladder hyper-reflexia both via agonist effects at both CB₁ and VR1 receptors and by potentiating anandamide by inhibition of its degradation, as a monotherapy.
- 15 20 Therefore the anti-spastic effect of arvanil was investigated.

METHODS

- Induction of CREA and assessment of spasticity was as described previously (14).
- 25 Capsaicin (VR-1) agonists, capsaizepine (VR-1) antagonists were purchase from RBI/sigma. SR141716A (abbreviated to SR-1. CB1 receptor antagonists), SR144528. The N-acyl-vanillyl-amides Arvanil and linvanil were synthesized as described previously (13)

30 RESULTS

- Following the accumulation of neurological deficits induced by autoimmune attack of the central nervous system induced during chronic relapsing experimental allergic encephalomyelitis, ABH mice develop spasticity (14). Arvanil when injected into animals induced a rapid amelioration of the spastic response, resistance to flexion of the hindlimb, when injected iv. at 0.1mg/kg ($P < 0.001$. Figures 10 and 11), 0.05mg/kg
- 35

($P < 0.001$) and 0.01mg/kg ($P < 0.01$). The maximal response being obtained within 10-30 min following injection (Figure 11). The efficacy was comparable to or better than the CB₁ agonist 5.0mg/kg methanandamide ($P < 0.001$ Figure 11) despite much lower doses being injected. Furthermore arvanil has a lower affinity/agonist activity *in vitro*.than methanandamide (13,14), which at a comparable low dose (0.05mg/kg iv.) failed to significantly inhibit spasticity compared to baseline levels (0.05mg/kg iv.). As arvanil is known to inhibit the anandamide transporter as well as VR-1 suggested that addition systems make be active in accounting for its potent activity. Linvanil was also active but consistent with its lower receptor binding affinities (13) this appeared less active than structurally related arvanil As blockage of the anandamide transporter can inhibit spasticity (15) and the transporter inhibitor AM404 can also bind to VR-1 (11), the potential of the vanilloid system to affect spasticity was investigated.

The pungent VR-1 agonists capsaicin (non CB₁.CB₂ binding) was injected into spastic animals at a maximal tolerated dose (0.1mg/kg iv). Surprisingly this induced a modest but nevertheless significant reduction ($P < 0.01$) in the degree of spasticity (Figures 11 and 12), peaking at approximately 30 minutes. Furthermore that this was mediated by VR-1 was supported following the inhibition of the anti-spastic effect using the VR-1 antagonist capsazepine (40mg/kg iv.) when injected 10-20 minutes prior to capsaicin (Figures 11 and 12). This suggests that VR-1 agonists may have some potential role in the treatment of spasticity. However in this instance VR-1 antagonism with capsazepine failed to affect the level of spasticity (Figures 11 and 12) which is in marked contrast to the rapid increase in spasticity following cannabinoid receptor antagonism (14,15). Following the injection of a combination of SR141716A (CB₁-selective antagonist 5mg/kg iv.) and SR144528 (CB₂-selective antagonist 5mg/kg iv.) tail and hindlimb spasticity increased however this rapidly returned ($P > 0.05$) to starting levels (14,15) within 20 minutes. If such pretreated animals were then injected with *R*(+)-WIN55,212 (5mg/kg i.p.) that is a CB₁ and CB₂ but not VR-1 agonist, then the anti-spastic effect of *R*(+)-WIN55,212 was eliminated. (Figure 12). Although CB antagonism could inhibit the anti-spastic effect of *R*(+)-WIN55,212 there was no readily apparent inhibition of this response when the same animals had been pretreated with capsazepine (Figure 12). This would be consistent with the lack activity of *R*(+)-WIN55 on the vanilloid system. Thus the data suggest that both the vanilloid and CB receptor systems may be targets for therapy of spasticity.

SUMMARY

Chronic relapsing experimental allergic encephalomyelitis (CREAE) is an
5 autoimmune model of multiple sclerosis (MS)¹. Although these diseases are typified
by relapsing-remitting paralytic episodes, following CREAE induction by sensitisation
to myelin antigens¹, Biozzi ABH mice also developed spasticity and tremor. These
symptoms also occur during MS and are difficult to control. This has prompted some
patients to self-medicate, and perceive benefit from cannabis use². While these
10 claims have been backed up by small clinical studies, mainly with non-quantifiable
outcomes³⁻⁷, the value of cannabis use in MS remains anecdotal.

In Example 1, we found that cannabinoid receptor (CB) agonism using: *R*(+)-WIN
55,212; Δ^9 -tetrahydrocannabinol (THC); methanandamide and JWH-133⁸
15 ameliorated quantitatively both tremor and spasticity in diseased mice. Importantly
the exacerbation of these signs following CB₁ and CB₂ receptor, notably CB₁
receptor, antagonism using SR14716A and SR144528⁸, suggests that the
endogenous cannabinoid system is tonically active in the control of tremor and
spasticity.

20

This provides a rationale for MS patient's indications for the therapeutic potential of
cannabis in the control of MS symptomology², and provides a means of evaluating
more selective cannabinoids in the future.

25 In addition, it is known that cannabinoid receptor (CB) agonism, including that
produced by injected naturally occurring cannabinoids, can limit spasticity in a
chronic relapsing experimental allergic encephalomyelitis (CR-EAE) model of
multiple sclerosis (MS)¹⁰¹. Importantly cannabinoid receptor antagonism transiently
exacerbated spasticity¹⁰¹.

30

Whilst the inverse agonist properties of these antagonist compounds¹⁰² may account
for these observations, alternatively they suggested tonic control of spasticity by the
endocannabinoid system. This was demonstrated in Example 2 following
amelioration of spasticity using inhibitors of endocannabinoid reuptake (AM404)¹⁰³ or
35 hydrolysis (AM364)¹⁰⁴. Importantly inhibition of the extracellular release of

endocannabinoids¹⁰⁵ by rolipram, a⁴⁸ phosphodiesterase inhibitor, resulted in the transient exacerbation of spasticity.

Thus is possible to control spasticity through manipulation of the endocannabinoid system without directly targeting the cannabinoid receptors themselves, and thus has implications for symptom control in MS, and other neuromuscular disease conditions.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be within the scope of the following claims.

GENERAL TEXT REFERENCES

1. Baker, D., *et al.* Induction of chronic relapsing experimental allergic encephalomyelitis in Biozzi mice. *J. Neuroimmunol.* 28, 261-270 (1990).
2. Consroe, P., Musty, R., Rein, J., Tillery, W. & Pertwee, R. The perceived effects of smoked cannabis on patients with multiple sclerosis. *Eur. Neurol.* 38, 44-48 (1997).
3. Consroe, P. Cannabinoid systems as targets for the therapy of neurological disorders *Neurobiol. Dis.* 5, 534-551 (1998).
4. Petro D.J. & Ellenberger, C. Treatment of human spasticity with Δ^9 -tetrahydrocannabinol. *J. Clin. Pharmacol.* 21(suppl.), 413s-416s (1981).
5. Clifford D.B. Tetrahydrocannabinol for tremor in multiple sclerosis. *Ann. Neurol.* 13, 669-671 (1983).
6. Ungerleider, J.T. Andyrsiak, T, Fairbanks, L., Ellison, G.W., & Myers, L.W. Δ^9 -THC in the treatment of spasticity associated with multiple sclerosis. *Adv. Alcohol Substance Abuse* 7, 39-50 (1987).

7. Martyn, C. N., Illis L. S. & Thom, J. Nabilone in the treatment of multiple sclerosis. *Lancet* **345**, 579 (1995).
8. Pertwee, R. G. Pharmacology of Cannabinoid Receptor Ligands, *Curr. Med. Chem.* **6**, 635-664 (1999).
- 5 9. Lyman, W. D., Sonett, J. R., Brosnan, C. F., Elkin, R., & Bornstein, M. B. Δ^9 -tetrahydrocannabinol: a novel treatment for experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 1989 Jun; **23**, 73-81 (1989).
10. Wirguin, I., *et al.* Suppression of experimental autoimmune encephalomyelitis by cannabinoids. *Immunopharmacology.* **28**, 209-214 (1994).
- 10 11. Heller, A. H., & Hallet, M. Electrophysiological studies with the spastic mutant mouse. *Brain Res.* **234**, 299-308 (1982).
12. Chai, C. K. Hereditary spasticity in mice. *J. Heredity* **52**, 241-243 (1961).
13. Pertwee R.G.. Pharmacology of cannabinoid CB1 and CB2 receptors *Pharmacol. Therapeut.* **74**, 129-180 (1997).
- 15 14. Breivogel, C.S. & Childers, S.R. The Functional Neuroanatomy of Brain Cannabinoid Receptors *Neurobiol. Dis.* **5**, 417-431 (1998).
15. D.J., Landsman, R.S., Burkey, T.H., Consroe, P., Roeske W.R. & Yamamura, H.I. SR141716A is an inverse agonist at the human cannabinoid CB1 receptor. *Eur. J. Pharmacol.* **334**, R1-R2 (1997).
- 20 16. Portier, M., *et al.* SR 144528, an antagonist for the peripheral cannabinoid receptor that behaves as an inverse agonist. *J. Pharmacol Exp. Ther.* 1999 Feb; **288**, 582-589 (1999).
17. Calignano, A., La Rana, G., Giuffrida, A., & Piomelli, D. Control of pain initiation by endogenous cannabinoids. *Nature.* **394**, 277-281 (1998).
- 25 18. Giuffrida, A., *et al.* Dopamine activation of endogenous cannabinoid signalling in dorsal striatum. *Nat. Neurosci.* **2**, 358-363 (1999).
19. Huffman, J.W. *et al.*, 3-(1',1'-Dimethylbutyl)-1-deoxy- Δ^8 -THC and Related Compounds: Synthesis of Selective Ligands for the CB2 Receptor. *Bioorg. Med. Chem.* in press
- 30 20. Noth, J. (1991) Trends in the pathophysiology and pharmacotherapy of spasticity. *J. Neurol.* **238**, 131-139 (1991).
101. Baker, D., *et al.* Cannabinoids control spasticity and tremor in a multiple sclerosis model. *Nature* March 2nd (2000).
- 35 102. Pertwee, R. G. Pharmacology of Cannabinoid Receptor Ligands, *Curr. Med. Chem.* **6**, 635-664 (1999).

103. Beltramo, M., *et al.* Functional role of high-affinity anandamide transport, as revealed by selective inhibition. *Science*. 277, 1094-1097. (1997).
104. Deutsch, D. G., *et al.* Fatty acid sulfonyl fluorides inhibit anandamide metabolism and bind to the cannabinoid receptor. *Biochem. Biophys. Res. Commun.* 231, 217-221 (1997)
105. Di Marzo, V. & De Petrocellis. The endogenous cannabinoid signalling system: chemistry, biochemistry and physiology. *Internet. J. Sci. Biol. Chem.* 1, www.netsci-journal.com/97v1/97007/index.htm (1997).
106. Giuffrida, A., *et al.* Dopamine activation of endogenous cannabinoid signalling in dorsal striatum. *Nat. Neurosci.* 2, 358-363 (1999).
107. Sommer, N. *et al.* The antidepressant rolipram suppresses cytokine production and prevents autoimmune encephalomyelitis. *Nat. Med.* 1, 244-8. (1995).
108. Genain, C.P. *et al.* Prevention of autoimmune demyelination in non-human primates by a cAMP-specific phosphodiesterase inhibitor. *Proc. Natl. Acad. Sci. USA.* 92, 3601-3605 (1995).
109. Consroe, P., Musty, R., Rein, J., Tillery, W. & Pertwee, R. The perceived effects of smoked cannabis on patients with multiple sclerosis. *Eur. Neurol.* 38, 44-48 (1997).
110. Wong, D.T., Bymaster, F.P., Engleman, E.A. Prozac (fluoxetine, Lilly 110140), the first selective serotonin uptake inhibitor and an antidepressant drug: twenty years since its first publication. *Life Sci.* 57, 411-441 (1995).
201. Consroe, P.(1998). Brain cannabinoid systems as targets for the therapy of neurological disorders. *Neurobiol. Dis.* 5, 534-551.
202. Pertwee. R.G. (1999) Pharmacology of cannabinoid receptor ligands. *Curr. Med. Chem.* 6, 635-664.
203. Baker, D., Pryce G., Croxford, J.L. Brown P, Pertwee, R.G., Huffman J.W. and Layward L. (2000), Cannabinoids control spasticity and tremor in a multiple sclerosis model. *Nature* 404, 84-87.
204. Baker D., O'Neill J.K., Gschmeissner S.E., Wilcox C.E., Butter C., and Turk J.L. (1990) Induction of chronic relapsing experimental allergic encephalomyelitis in Biozzi mice. *J. Neuroimmunol* 28, 261-270.

205. Di Marzo, V., Melck, D., Bisogno, T., and De Petrocellis, L. (1998) Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. *Trends Neurosci.* 21, 521-528.
206. Preece, N.E., Amor, S., Baker, D., Gadian, D.G., O'Neill, J.K. and Urenjak, J. (1994) Experimental encephalomyelitis modulates inositol and taurine in the spinal cord of Biozzi mice. *Mag. Res. Med.* 32, 92-97.
207. Bisogno, T., Berrendero, F., Ambrosino, G., Cebeira, M., Ramos, J.A., Fernandez-Ruiz, J.J. and Di Marzo, V. (1999) Brain regional distribution of endocannabinoids: implications for their biosynthesis and biological function. *Biochem. Biophys. Res. Commun.* 256, 377-380.
208. Di Marzo, V., Berrendero, F., Bisogno, T., Gonzalez, S., Cavaliere, P., Romero, J., Cebeira, M., Ramos, J.A. and Fernandez-Ruiz, J.J. (2000). Enhancement of anandamide formation in the limbic forebrain and reduction of endocannabinoid contents in the striatum of Δ^9 -tetrahydrocannabinol-tolerant rats. *J. Neurochem.* 74, 1627-1635.
209. Beltramo, M., Stella, N., Calignano, A., Lin, S.Y., Makriyannis, A. and Piomelli, D. (1997). Functional role of high-affinity anandamide transport, as revealed by selective inhibition. *Science*, 277, 1094-1097.
210. Deutsch, D.G., Lin, S., Hill, W.A., Morse, K.L., Salehani, D., Arreaza, G., Omeir, R. L., and Makriyannis, A. (1997). Fatty acid sulfonyl fluorides inhibit anandamide metabolism and bind to the cannabinoid receptor. *Biochem. Biophys. Res. Commun.* 231, 217-221.
211. De Petrocellis, L., Bisogno, T., Davis, J.B., Pertwee, R.G., and Di Marzo V. (2000) Overlap between the ligand recognition properties of the anandamide transporter and the VR-1 vanilloid receptor: inhibitors of anandamide uptake with negligible capsaicin-like activity. *FEBS Lett.* 483, 52-56.
212. Giuffrida, A., Parsons, L. H., Kerr, T. M., Rodriguez de Fonseca, F., Navarro, M., and Piomelli, D. (1999) Dopamine activation of endogenous cannabinoid signaling in dorsal striatum. *Nat. Neurosci.* 2, 358-363.
213. Leweke, F.M., Giuffrida, A., Wurster, U., Emrich, H.M., and Piomelli, D. (2000) Elevated endogenous cannabinoids in schizophrenia. *Neuroreport* 10, 1665-1669.

214. Bisogno, T. Sepe, N., Melck, D., Maurelli, S., De Petrocellis, L. and Di Marzo, V. (1997) Biosynthesis, release and degradation of the novel endogenous cannabimimetic metabolite 2-arachidonoylglycerol in mouse neuroblastoma cells. *Biochem. J.*, 322, 671-677.
- 5 215. Di Marzo, V., Bisogno, T., Sugiura, T., Melck, D., and De Petrocellis, L. (1998) The novel endogenous cannabinoid 2-arachidonoylglycerol is inactivated by neuronal- and basophil-like cells: connections with anandamide. *Biochem. J.* 331, 15-19
216. Maccarrone, M., Bari, M., Lorenzon T., Bisogno T., Di Marzo V. and Finazzi-Agro, A. (2000). Anandamide uptake by human endothelial cells and its regulation by nitric oxide. *J. Biol. Chem.* 275, 13484-13492.
- 10 217. Felder, C. C., Nielsen, A., Briley, E.M., Palkovits, M., Priller, J., Axelrod, J., Nguyen, D. N., Richardson, J.M., Riggin, R. M., Koppel, G.A., Paul, S.M., and Becker, G. W. (1996). Isolation and measurement of the endogenous cannabinoid receptor agonist, anandamide, in brain and peripheral tissues of human and rat. *FEBS. Letts.* 393, 231-235.
218. Zygmunt, P.M., Petersson, J., Andersson, D.A., Chuang, H., Sorgard M., Di Marzo, V., Julius, D. and Hogestatt E.D. (1999). Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature*, 400, 452-447 .
- 20 219. Smart D and Jerman J.C. (2000). Anandamide: an endogenous activator of the vanilloid receptor. *Trends Pharmacol. Sci.* 21, 134.
220. Di Marzo, V., Hill M. P., Bisogno, T., Crossman A.R. and Brotchie J.M. (2000) Enhanced levels of endocannabinoids in the globus pallidus are associated with a reduction in movement in an animal model of Parkinson's disease. *FASEB. J.* 14, 1432-1438
- 25 221. Abadji, V., Lin, S., Taha, G., Griffin, G., Stevenson, L. A., Pertwee, R.G., and Makriyannis, A. (1994). (*R*)-methanandamide: a chiral novel anandamide possessing higher potency and metabolic stability. *J. Med. Chem.* 37, 1889-1893.
222. Calignano, A., La Rana, G., Giuffrida, A. and Piomelli, D. (1998) Control of pain initiation by endogenous cannabinoids. *Nature* 394, 277-281.
- 30 223. De Petrocellis, L., Melck, D., Bisogno, T. and Di Marzo, V. (2000) Endocannabinoids and fatty acid amides in cancer, inflammation and related disorders. *Chem. Phys. Lipids*, In press.

224. Mechoulam, R., Fride, E., and Di Marzo, V. (1998). Endocannabinoids. *Eur J Pharmacol.* 359, 1-18.
225. Goparaju, S. K., Ueda, N., Yamaguchi, H., and Yamamoto, S. (1999) Anandamide amidohydrolase reacting with 2-arachidonoylglycerol, another cannabinoid receptor ligand. *FEBS Letts.* 422, 69-73.
226. Gifford, A.N., Bruneus, M., Lin, S.Y., Goutopoulos, A., Makriyannis, A. Volkow, N.D., and Gatley, S.J. (1999) Potentiation of the action of anandamide on hippocampal slices by the fatty acid amide hydrolase inhibitor, palmitylsulphonyl fluoride (AM374). *Eur. J. Pharmacol.* 383, 9-14.
- 10 227. Pertwee, R.G. (2000) Cannabinoid receptor ligands: clinical and neuropharmacological considerations relevant to future drug discovery and development. *Expert. Opin. Invest. Drugs.* In press.
228. Fowler, C.J., Jewkes, D., McDonald, W.I., Lynn, B. and de Groat, W.C. (1992) Intravesical capsaicin for neurogenic bladder dysfunction. *Lancet* 339 (8803), 1239.
- 15 229. Coutts, A.A. and Pertwee R.G. (1998) Evidence that cannabinoid-induced inhibition of electrically evoked contractions of the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine can be modulated by Ca^{2+} and cAMP. *Can. J. Physiol. Pharmacol.* 76, 340-346.
230. Sommer, N., Loschmann P.A., Northoff G.H., Weller, M., Steinbrecher A., Steinbach, J.P., Lichtenfels, R., Meyermann R., Riethmuller A., Fontana, A., Dichgans, J. and Martin, R. (1995). The antidepressant rolipram suppresses cytokine production and prevents autoimmune encephalomyelitis. *Nat. Med.* 1, 244-248.
231. Aceto, M.D., Scates, S.M., Razdan, R.K. and Martin, B.R. (1998) Anandamide, an endogenous cannabinoid, has a very low physical dependence. *J. Pharmacol. Exp. Ther.* 287, 598-605.
- 25

REFERENCES FOR CB1 SECTION

1. Gerard, C. M.; Mollereau, C.; Vassart, G.; Parmentier, M. : Molecular cloning of a human cannabinoid receptor which is also expressed in testis. *Biochem. J.* 279: 129-134, 1991.
- 30

2. Hoehe, M. R.; Caenazzo, L.; Martin z, M. M.; Hsieh, W.-T.; Modi, W. S.; Gershon, E. S.; Bonner, T. I. : Genetic and physical mapping of the human cannabinoid receptor gene to chromosome 6q14-q15. *New Biologist* 3: 880-885, 1991.

5

3. Ledent, C.; Valverde, O.; Cossu, G.; Petitet, F.; Aubert, J.-F.; Beslot, F.; Bohme, G. A.; Imperato, A.; Pedrazzini, T.; Roques, B. P.; Vassart, G.; Fratta, W.; Parmentier, M. : Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB(1) receptor knockout mice. *Science* 283: :401-404, 1999.

10

4. Matsuda, L. A.; Lolait, S. J.; Brownstein, M. J.; Young, A. C.; Bonner, T. I. : Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 346: 561-564, 1990.

15

5. Modi, W. S.; Bonner, T. I. : Localization of the cannabanoid receptor locus using non-isotopic in situ hybridization. *Cytogenet. Cell Genet.* 58: 1915 only, 1991.

REFERENCE FOR CB2 SECTION

20

1. Munro, S.; Thomas, K. L.; Abu-Shaar, M. : **Molecular characterization of a peripheral receptor for cannabinoids.** *Nature* 365: 61-65, 1993.

REFERENCES FOR VANILLOID SECTION

25

1. Bartoshuk, L. M.; Duffy, V. B.; Miller, I. J. :

PTC/PROP tasting: anatomy, psychophysics, and sex effects. *Physiol. Behav.* 56: 1165-1171, 1994.

30

2. Caterina, M. J.; Leffler, A.; Malmberg, A. B.; Martin, W. J.; Trafton, J.; Petersen-Zeltz, K. R.; Koltzenburg, M.; Basbaum, A. I.; Julius, D. :

Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* 288: 306-313; 2000.

35

3. Caterina, M. J.; Schumacher, M. A.; Tominaga, M.; Rosen, T. A.; Levine, J. D.; Julius, D. :

The capsaicin receptor: a heat-activated ion channel in the pain pathway.

Nature 389: 816-824, 1997.

4. Liu, L.; Simon, S. A. :

Similarities and differences in the currents activated by capsaicin, piperine, and zingerone in rat trigeminal ganglion cells. *J. Neurophysiol.* 76: 1858-1869, 1996.

5. Prescott, J.; Stevenson, R. J. :

Psychophysical responses to single and multiple presentations of the oral irritant zingerone: relationship to frequency of chili consumption. *Physiol. Behav.* 60: 617-624, 1996.

6. Stevenson, R. J.; Prescott, J. :

The effects of prior experience with capsaicin on ratings of its burn. *Chem. Senses* 19: 651-656, 1994.

7. Stevenson, R. J.; Yeomans, M. R. :

Differences in ratings of intensity and pleasantness for the capsaicin burn between chili likers and non-likers: implications for liking development. *Chem. Senses* 18: 471-482, 1993.

REFERENCES FOR EXAMPLE 4

1. Caterina M 1997 The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389:816-24.

2. Nagy I 1999 Noxious heat activates all capsaicin-sensitive and also a sub-population of capsaicin-insensitive dorsal root ganglion neurons. *Neuroscience* 88:995-7.

3. Chancellor M 1999 Intravesical capsaicin and resiniferatoxin therapy: spicing up the ways to treat the overactive bladder. *J Urol* 162:3-11.

4. Sindrup S 1999 Efficacy of pharmacological treatments of neuropathic pain: an update and effect related to mechanism of drug action. *Pain* 83:389-400.

5. Rice A. 2000 Local neuro-immune interactions in visceral hyperalgesia: bradykinin, neurotrophins and cannabinoids. (In Press).

6. Di Marzo V 1998 Biochemistry of the endogenous ligands of cannabinoid receptors. *Neurobiology of Disease* 5:386-404.

7. Farquhar-Smith W. 2000 Cannabinoid CB1 receptor expression in rat spinal cord. *Mol Cell Neurosci* 15:510-21.
8. Jaggar S. 1998 The anti-hyperalgesic actions of the cannabinoid anandamide and the putative CB2 agonist palmitoylethanolamide investigated in models of visceral and somatic inflammatory pain. *Pain* 76:189-99.
9. Rice A. 2000 Endocannabinoids prevent the referred viscerosomatic hyperalgesia associated with inflammation of the rat urinary bladder. *ICRS abstracts* 84
10. Zygmunt P. 1999 Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* 400:452-7.
11. Smart D. 2000 The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br J Pharmacol* 129:227-30.
12. Di Marzo V. 1998 Interactions between synthetic vanilloids and the endogenous cannabinoid system. *FEBS Lett* 436:449-54.
13. Melck D. 1999 Unsaturated long chain N-acyl-vanillyl-amides (N-AVAMs): Vanilloid receptor ligands that inhibit anandamide-facilitated transport 262:275-84.
14. Baker D. 2000 Cannabinoids control spasticity and tremor in a multiple. *Nature* 404:84-87
15. Baker D. 2000 Endocannabinoid control of spasticity. *FASEB J* In press

CLAIMS

1. Use of a modulator of an endocannabinoid in the manufacture of a medicament to treat a muscular disorder.
2. Use according to claim 1 wherein said modulator inhibits the reuptake of an endocannabinoid.
3. Use according to claim 1 or claim 2 wherein said modulator does not substantially agonise CB1.
4. Use according to any one of the preceding claims wherein said muscular disorder is a neuromuscular disorder.
5. Use of a modulator of endocannabinoid in the manufacture of a medicament to control spasticity and tremors.
6. A method of treatment comprising administering to a subject suffering from a muscular disorder a modulator of endocannabinoid and in such an amount to treat said muscular disorder.
7. Method according to claim 6 wherein said modulator inhibits the reuptake of an endocannabinoid.
8. Method according to claim 6 or claim 7 wherein said modulator does not substantially agonise CB1.
9. Method according to any one of claims 6 to 8 wherein said muscular disorder is a neuromuscular disorder.
10. A method of treatment comprising administering to a subject suffering from a muscular disorder a modulator of endocannabinoid and in such an amount to control spasticity and tremors.
11. A modulator of endocannabinoid to treat a muscular disorder.

12. A pharmaceutical composition comprising a modulator of endocannabinoid and optionally a pharmaceutically active carrier, diluent or excipient, said composition for subsequent use to treat a muscular disorder.

5

13. The invention according to any one of claims 1 to 12 wherein said modulator is a modulator of a vanilloid receptor and/or said modulator is used in conjunction with a modulator of a vanilloid receptor.

10

14. Use of a modulator of a vanilloid receptor in the manufacture of a medicament to treat a muscular disorder, preferably wherein said muscular disorder is a neuromuscular disorder.

15

15. Use of a modulator of a vanilloid receptor in the manufacture of a medicament to control spasticity and tremors.

16. A method of treatment comprising administering to a subject suffering from a muscular disorder a modulator of a vanilloid receptor and in such an amount to treat said muscular disorder, preferably a neuromuscular disorder.

20

17. A method of treatment comprising administering to a subject suffering from a muscular disorder a modulator of a vanilloid receptor and in such an amount to control spasticity and tremors.

25

18. A modulator of a vanilloid receptor to treat a muscular disorder.

19. A pharmaceutical composition comprising a modulator of a vanilloid receptor and optionally a pharmaceutically active carrier, diluent or excipient, said composition for subsequent use to treat a muscular disorder.

30

1 / 10

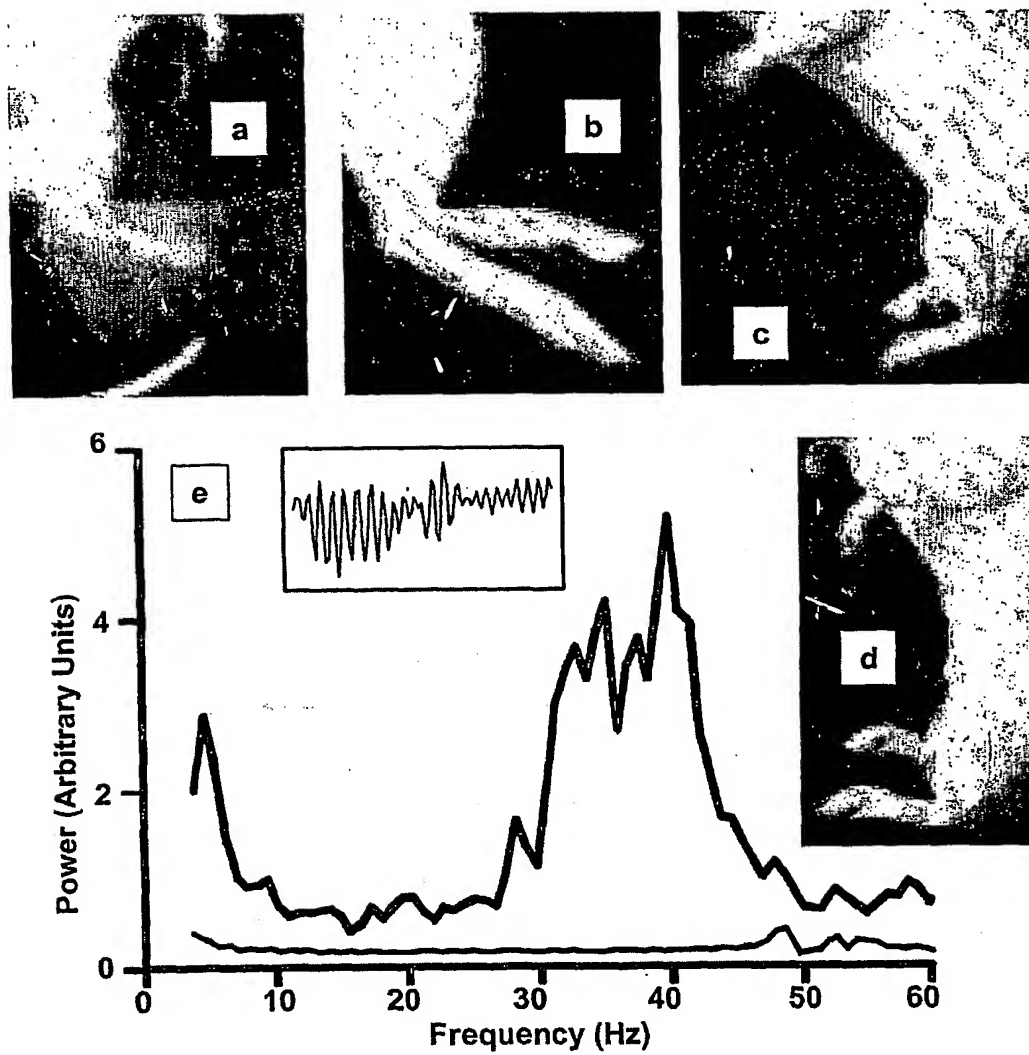


FIG. 1

2 / 10

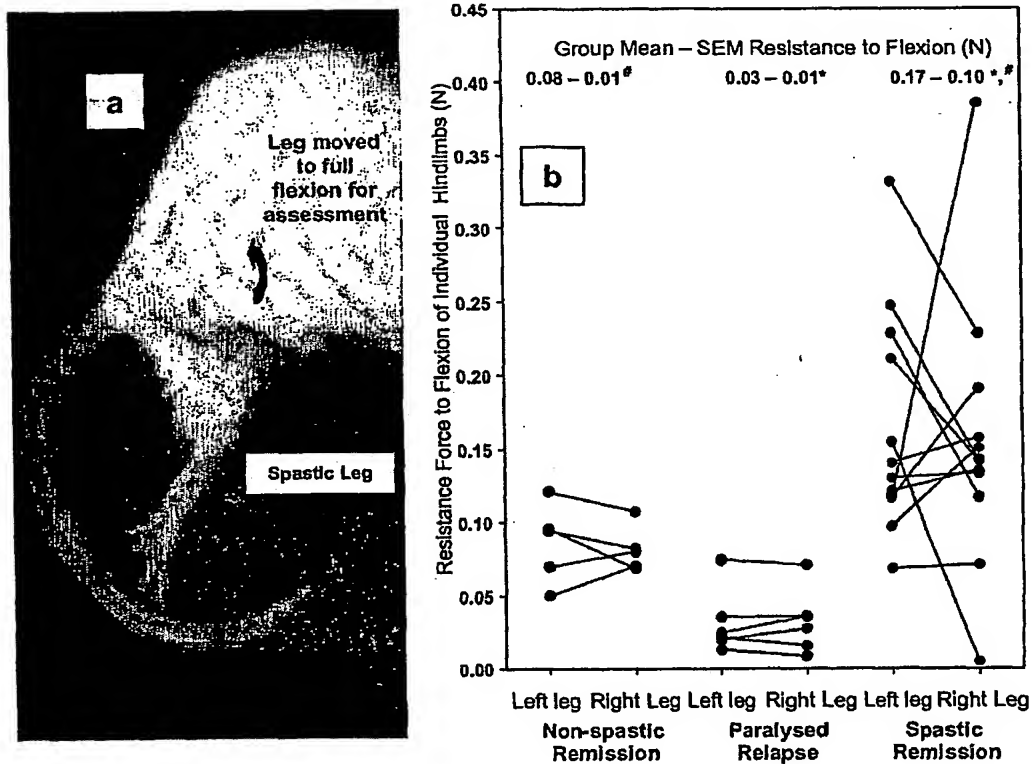


FIG. 2

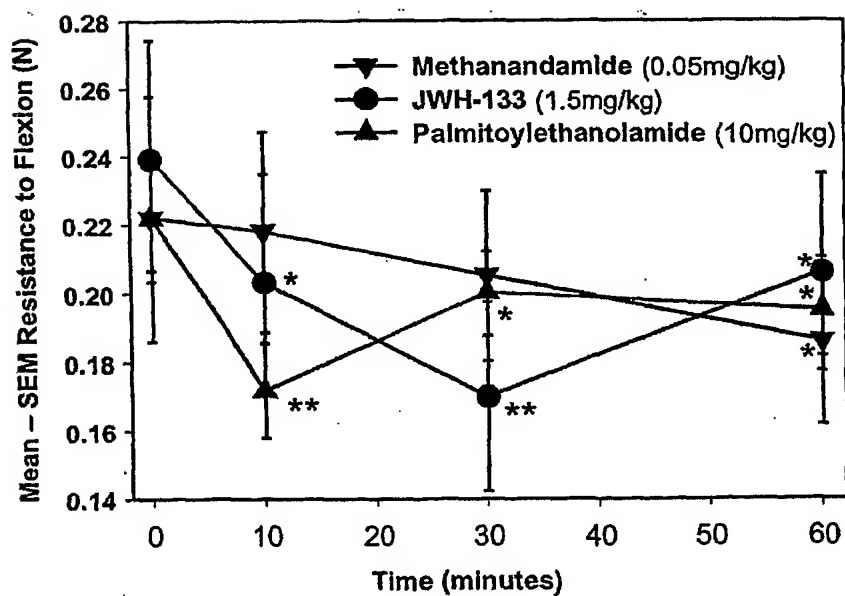


FIG. 4

3 / 10

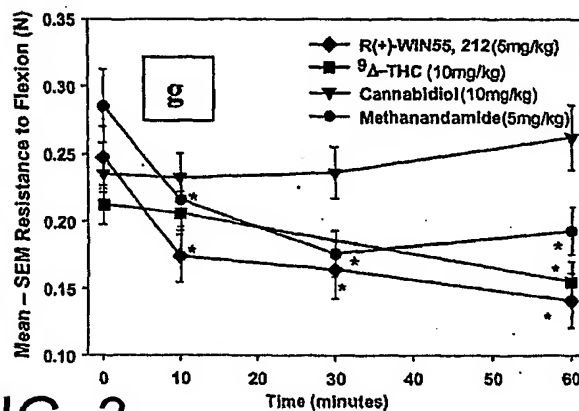
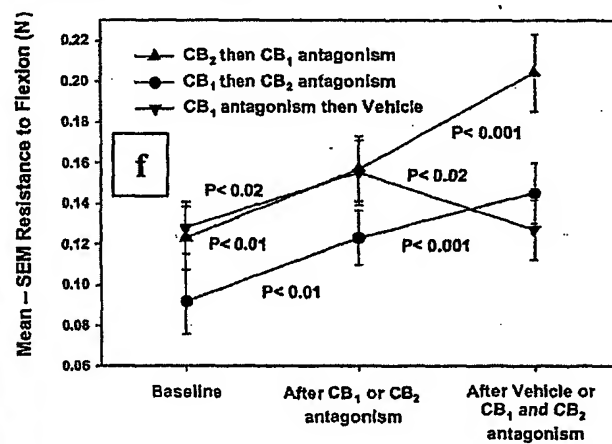
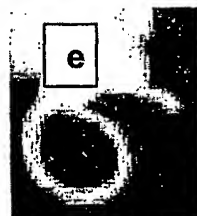
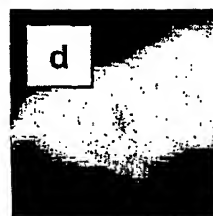
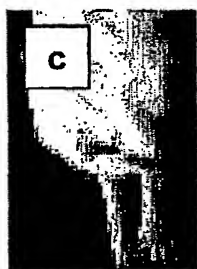
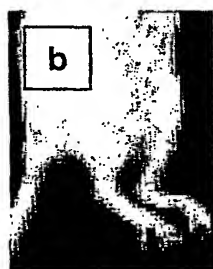
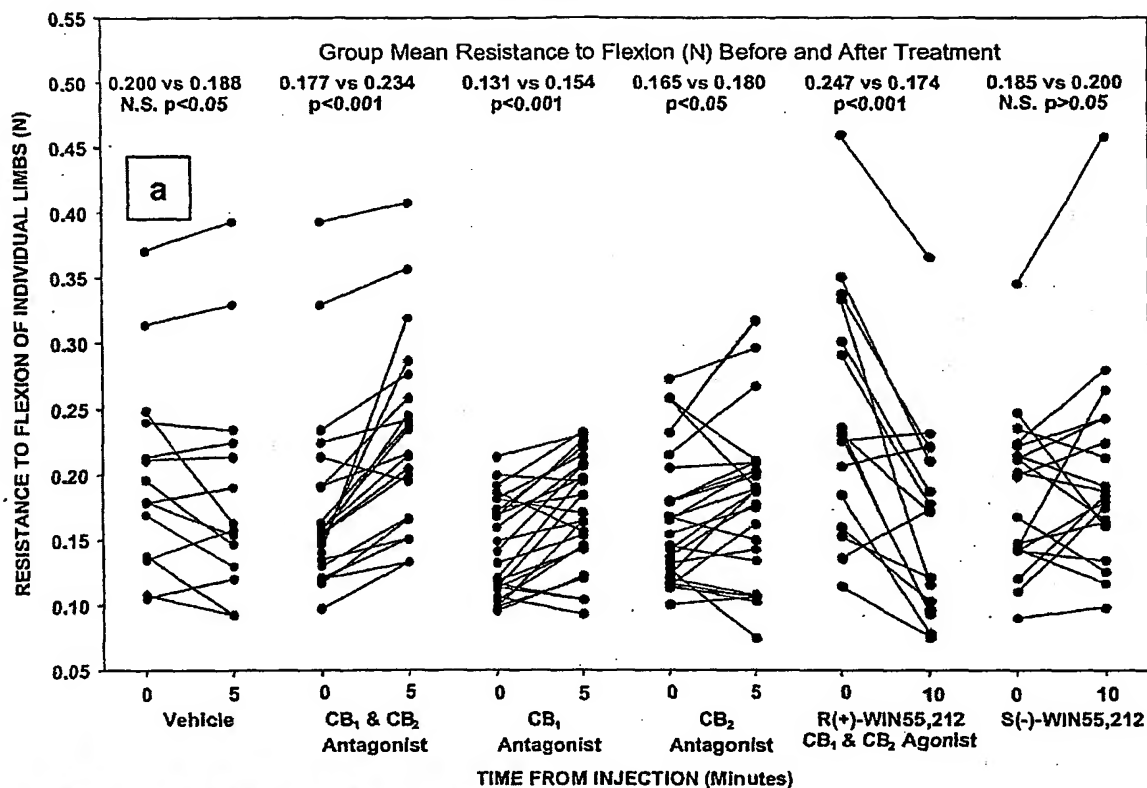


FIG. 3

SUBSTITUTE SHEET (RULE 26)

4 / 10

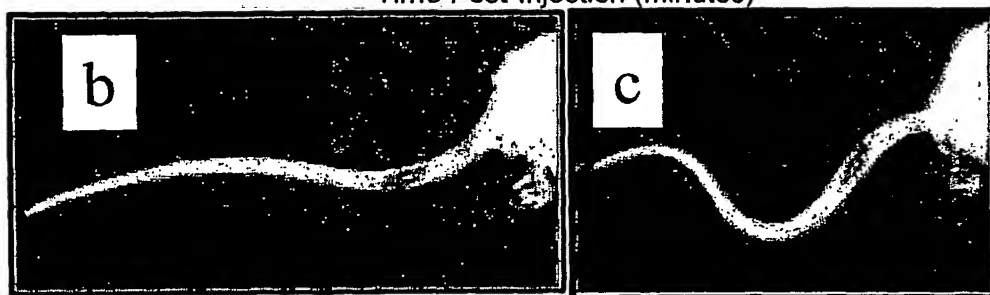
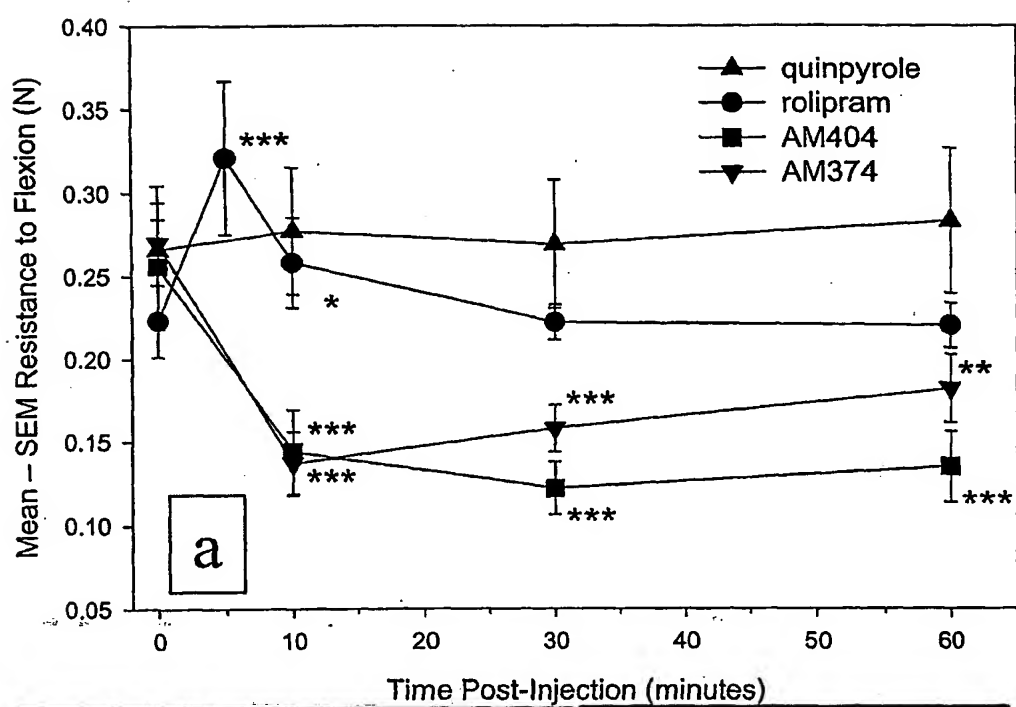


FIG. 5

5 / 10

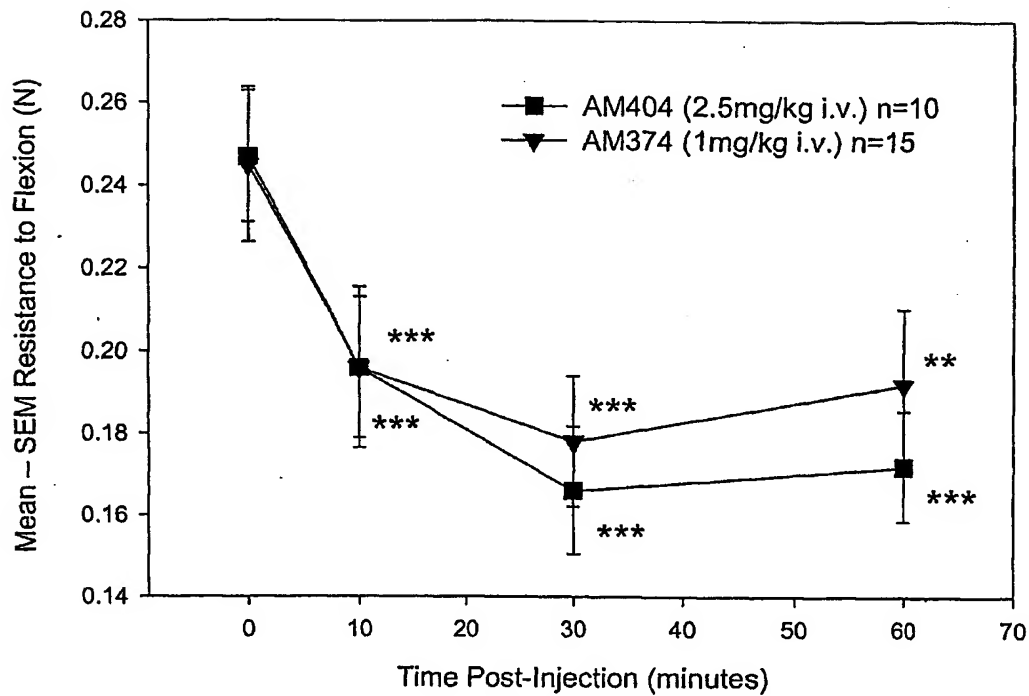


FIG. 6

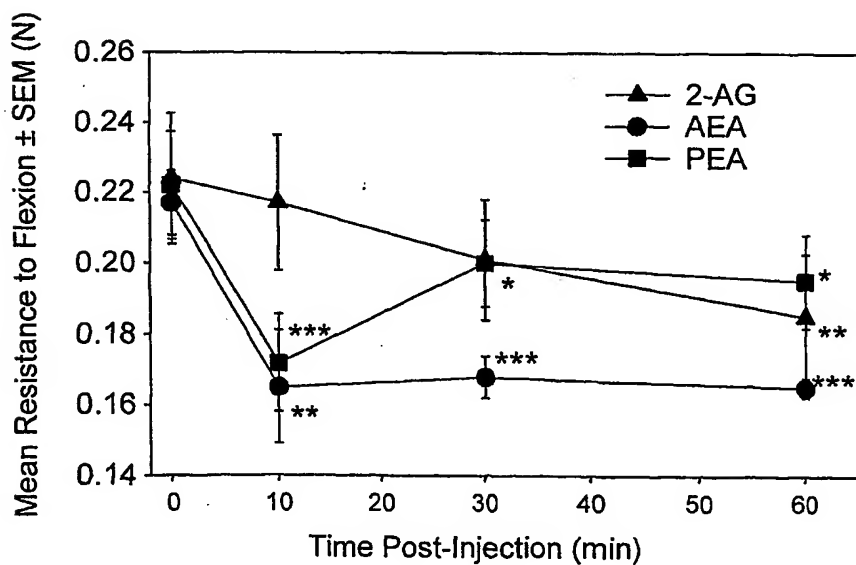


FIG. 8

6 / 10

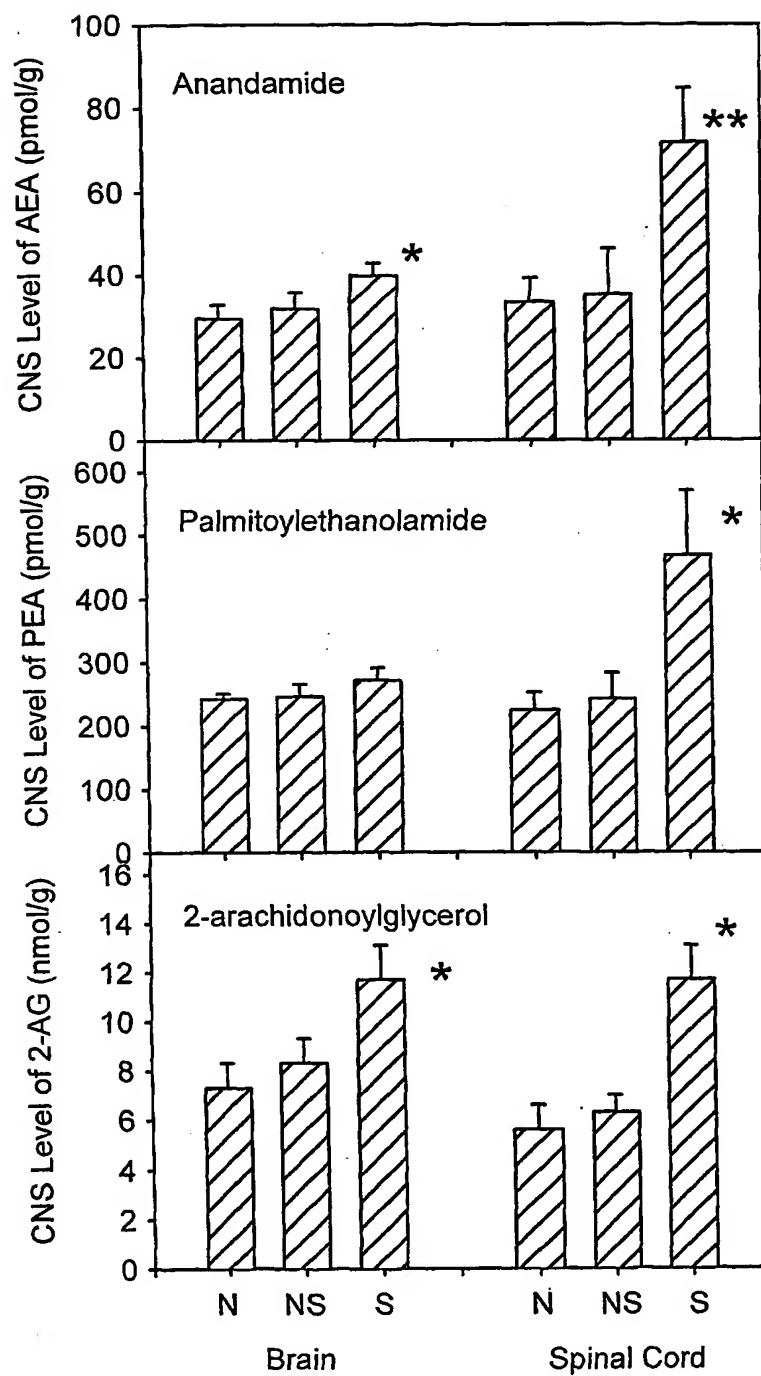


FIG. 7

7 / 10

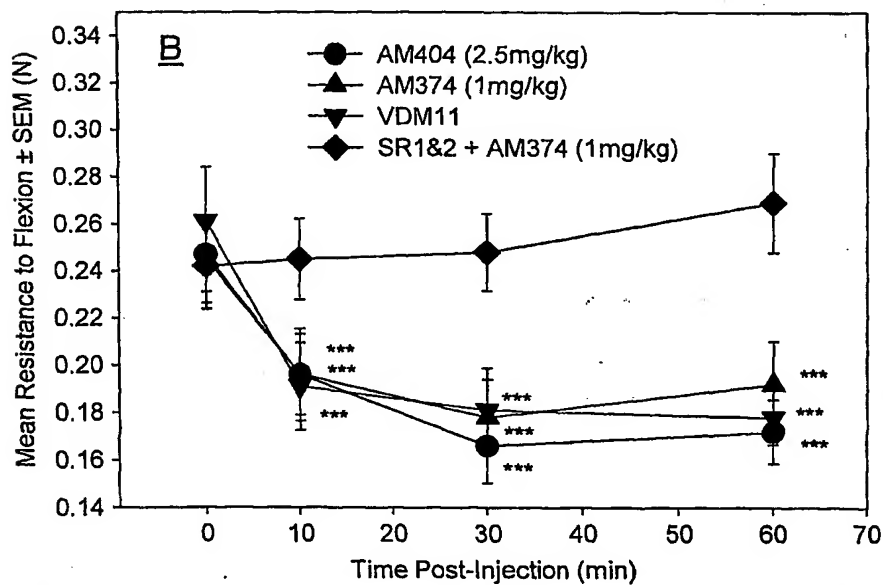
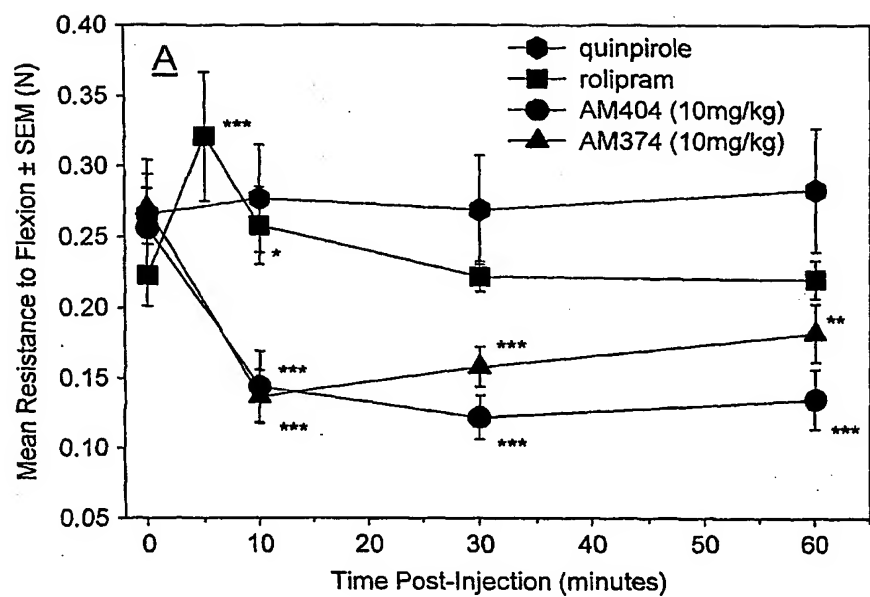


FIG. 9

8 / 10

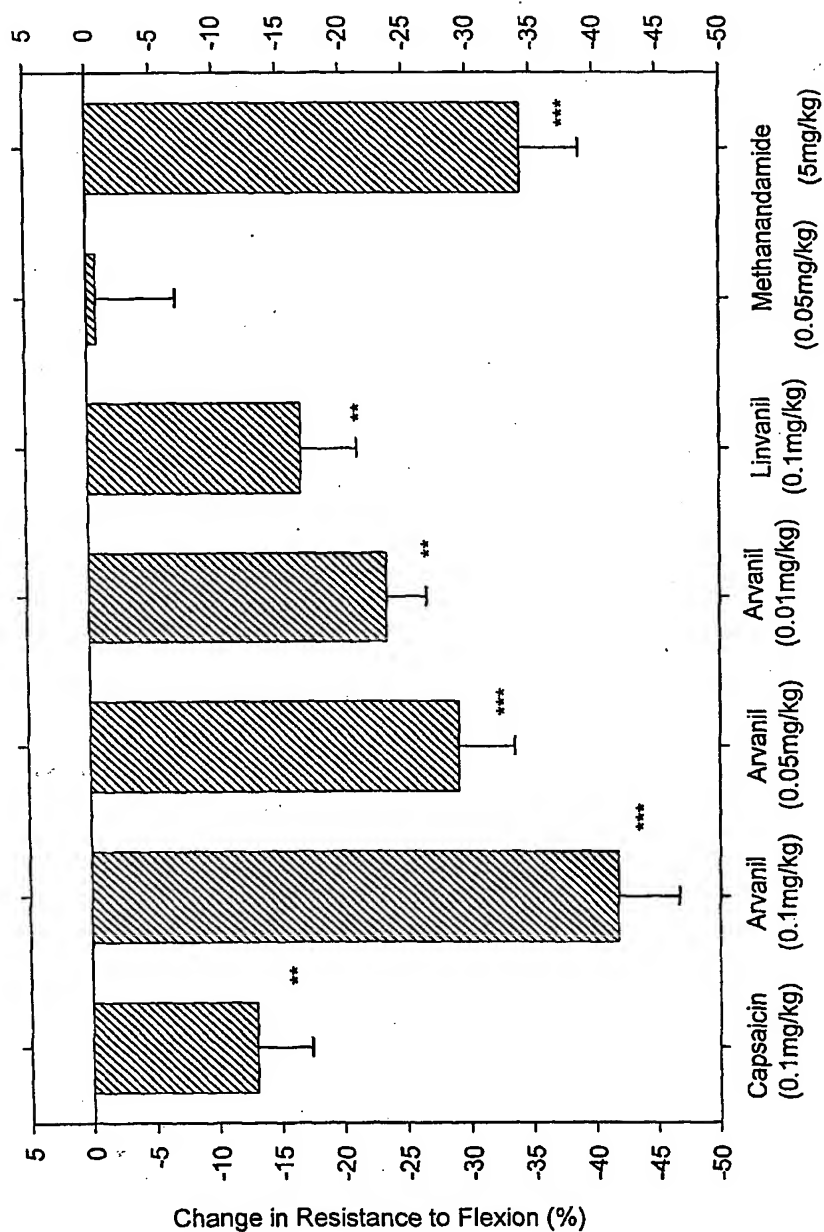


FIG. 10

9 / 10

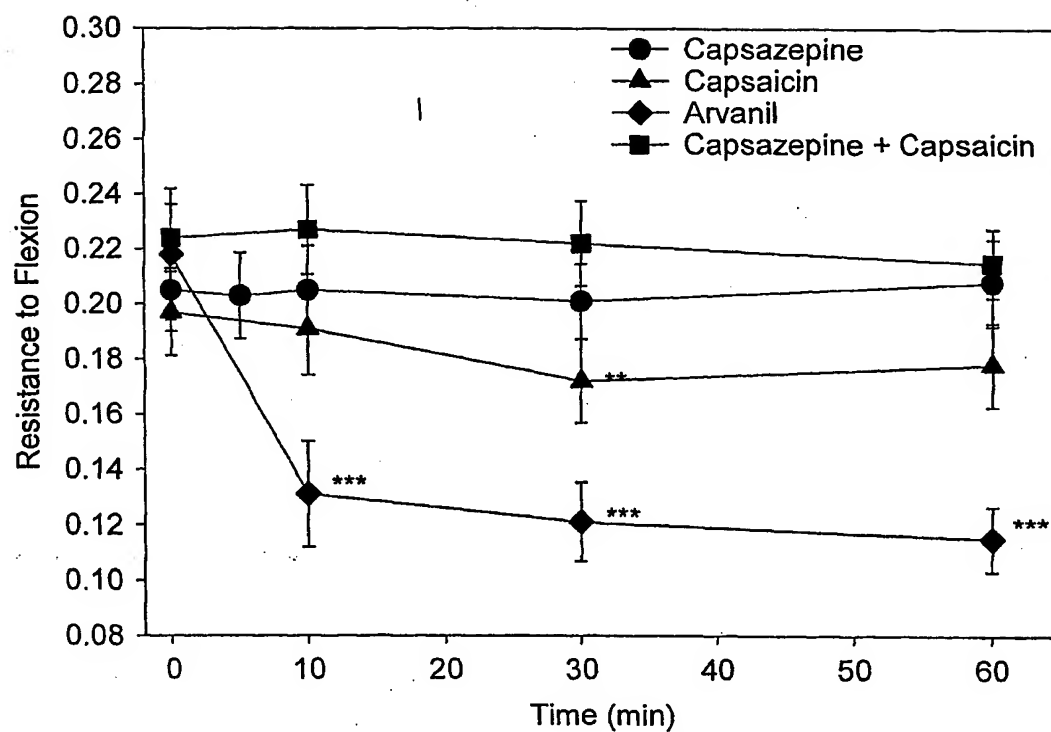


FIG. 11

10 / 10

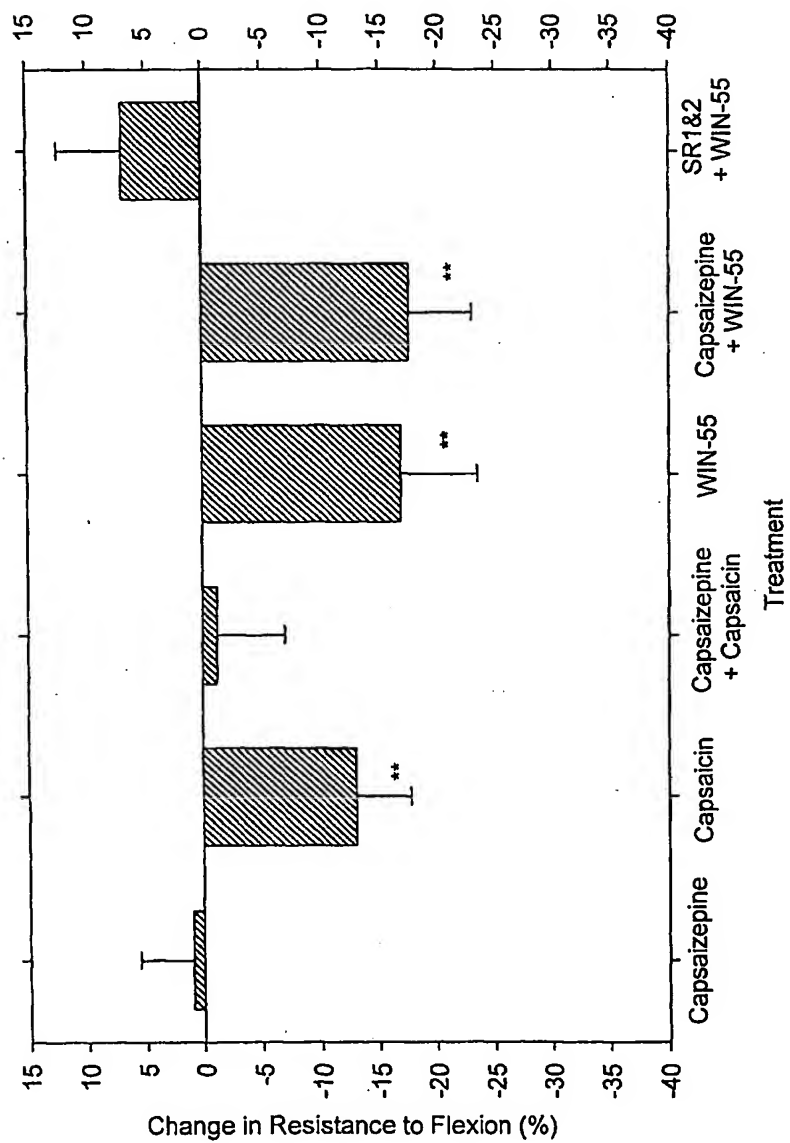


FIG. 12

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 01/00858

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/23

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EP0-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data, EMBASE, PHARMAPROJECTS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 198 37 638 A (BAYER AG) 24 February 2000 (2000-02-24)	1-13
Y	*cf. abstract, page 1, lines 1-9, page 18, lines 18-37*	14-19
X	WO 00 24362 A (UNIV VIRGINIA COMMONWEALTH) 4 May 2000 (2000-05-04)	1-13
Y	*cf. abstract, page 1, 1st para. and page 15, 3rd para.*	14-19
A	V. DI MARZO ET AL.: "Neurobehavioral in mice of N-vallinyl-arachidonyl-amide" EUROPEAN JOURNAL OF PHARMACOLOGY, vol. 406, 2000, pages 363-374, XP001007385 *cf. abstract, page 372, right-handed col., 2nd para.*	1-19

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

G document member of the same patent family

Date of the actual completion of the international search

31 July 2001

Date of mailing of the international search report

13/08/2001

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Stoltner; A

1

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 01/00858

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 99 64389 A (GOUTOPOULOS ANDREAS ; LIN SONYUAN (US); MAKRIYANNIS ALEXANDROS (US)) 16 December 1999 (1999-12-16) *cf. page 3, line 18 bridging with page 19, lines 1-29* -----	1-19
Y	US 4 681 897 A (BRAND LARRY M) 21 July 1987 (1987-07-21) *cf. abstract, col. 1, lines 9-39, col. 2, lines 18-22* -----	1-19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 01/00858

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 19837638 A	24-02-2000	AU 5420399 A WO 0010967 A EP 1105370 A	14-03-2000 02-03-2000 13-06-2001
WO 0024362 A	04-05-2000	AU 2143000 A	15-05-2000
WO 9964389 A	16-12-1999	EP 1084098 A	21-03-2001
US 4681897 A	21-07-1987	AU 571933 B AU 3764385 A CA 1241604 A DE 3574934 D DE 3586545 A DE 3586545 T DK 19385 A EP 0149545 A EP 0306060 A GB 2153672 A,B HK 59590 A IE 58335 B IE 58314 B JP 1996682 C JP 7017501 B JP 60208913 A NZ 210838 A PH 22723 A SG 44990 G US 4812446 A ZA 8500352 A	28-04-1988 24-07-1986 06-09-1988 01-02-1990 24-09-1992 04-03-1993 17-07-1985 24-07-1985 08-03-1989 29-08-1985 10-08-1990 08-09-1993 08-09-1993 08-12-1995 01-03-1995 21-10-1985 30-06-1988 28-11-1988 17-08-1990 14-03-1989 27-11-1985